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(54) Title: DIAGNOSIS FOR PORCINE MALIGNANT HYPERTHERMIA


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(57) Abstract

A purified DNA molecule comprises a DNA sequence of approximately 15.1 kb coding for normal or mutant RYR1 protein having a molecular weight of approximately 564,740 daltons. The DNA molecule has an endonuclease restriction map of Figure 1 and a sequence of Figure 2.

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DIAGNOSIS FOR PORCINE MALIGNANT HYPERTHERMIA

This invention relates to the animal disease
malignant hyperthermia (MH), to the cloning and
characterization of a gene associated with MH in swine,
5   and to the development of DNA and antibody-based methods
for detecting individual pigs susceptible to MH.

Malignant hyperthermia (MH) is a hypermetabolic
myopathy which is triggered in genetically-susceptible
human and animal individuals by potent, volatile
10   anesthetics such as halothane [Denborough and Lovell
(1960) *Lancet* 2, 545: Harrison et al. (1968) *Brit. Med.
J.* 3:594-595] or by depolarizing muscle relaxants such as
succinycholine [Harrison et al. (1969) *Brit. J.
Anaesthesia* 41:844-855]. In swine, it is also referred
15   to as porcine stress syndrome [Topel (1968) *Mod. Vet.
Pract.* 49:40-41; 59-60] because it may be triggered by
exertional [Ludvigsen (1953) *Internat. Vet. Congr.*
Stockholm 1:602-606], thermal [Forrest et al. (1968) *J.
of Appl. Physiol.* 24:33-39], anoxic [Lister et al. (1970)
20   *Am. J. Physiol.* 218:102-107], or mechanical [Gronert
(1980) *Anaesthesiol.* 44:36-43] stressors as well as
anesthesia [Hall et al (1966) *Brit. Med. J.* 4:1305]. A
similar stress syndrome may occur in MH-susceptible (MHS)
humans [Wingard (1974) *Lancet* 2;1450-1451] and dogs
25   [O'Brien et al. (1983) *Can. Vet. J.* 24:172-177]. MH is
characterized by the peracute development of contracture
and maximal rate of metabolism in muscle. These have
been proposed to occur due to an uncontrollable and
sustained elevation of myoplasmic calcium [Britt and
30   Kalow (1970) *Can. Anesthetists Soc. J.* 17:316-330: Lopez
et al. (1985) *Biophys. J.* 47:313a], which is known to
activate the contractile apparatus and metabolic
machinery of skeletal muscle [Martonosi (1984) *Physiol.
Rev.* 64:1240-1319).

35   The hyperactivity of muscle which occurs during MH
results in the depletion of ATP and glycogen stores and
the excessive formation of carbon dioxide, lactic acid

and heat. This thermogenesis, in conjunction with peripheral vasoconstriction, leads to hyperthermia. The rapid rate of aerobic metabolism, by depleting blood oxygen, causes cyanosis [Gronert (1980) *Anaesthesiol.* <u>53</u>:

5 395-423]. During MH, glycogenolysis, rhabdomyolysis and acidemia cause the release of large amounts of potassium from muscle and liver [Hall et al. (1980) *Brit. J. Anaesthesiol.* <u>52</u>:11-17] into the vascular compartment. The resultant hyperkalemia contributes to the development

10 of cardiac dysrhythmia and subsequent heart failure [Britt (1983) in "Complications in Anesthesiology" *F.K. Orkin and L.H. Cooperman (eds) Lippincott*, pp. 290-313].

Major revenue loss in the swine, pork and bacon industries occurs because of stress-induced MH deaths,

15 usually during transport to the slaughterhouse. This occurs in homozygotes which make up 1 to 2% of swine and is referred to as porcine stress syndrome (PSS). Greater loss occurs due to the development of inferior quality meat after slaughter of MHS homozygote or heterozygote

20 swine, which make up 10 to 30% of the population.

Activities and environmental stressors, which may trigger MH, include transport, restraint, mating, farrowing, fighting, vigorous exercising, and hot, humid weather [Mitchell and Heffron (1982) *Adv. Food Res.*

25 <u>28</u>:167-230]. The neural stimulation of muscle which occurs during slaughter [McLoughlin (1971) in "Condition and Meat Quality of Pigs" *G.R. Hersel-de-heer et al (eds) Pudoc*, pp123-132], and the anoxia [Lister et al (1970) *Am. J. Physiol.* <u>215</u>:102-107] which occurs with cardiac

30 failure, are sufficient to trigger hypermetabolism in MHS muscle.

As a result of the excessive rates of production of lactic acid and heat[Lawrie (1960) *J. Comp. Pathol.* <u>70</u>:273-295], sarcoplasmic proteins denature, thereby

35 causing a deterioration of the water-binding capacity of muscle. Furthermore, the increased osmotic activity due to end-products of hypermetabolism causes an influx of

water from the extracellular space, thereby resulting in hemoconcentration and increased intramyofiber water content [Berman et al (1970) *Nature* 220:653-655]. The muscle becomes pale, soft and exudative, sour-smelling

5 and loose-textured [Briskey (1964) *Adv. in Food Res.* 13:89-178]. The shrinkage due to water loss during storage, transport an¹ processing of the carcass is the major cause of wholesale losses at pork packing plants [Smith and Lesser (1982) *Anim. Prod.* 34:291-299].

10 Shorter shelf-life and decreased organoleptic acceptability contribute to retail losses. Other causes of lost revenue with MHS swine are their decreased average daily weight gain, conception rates, litter sizes and boar breeding performance [Webb (1980) *Vet. Record*

15 106:410-412; Carden et al (1985) *Anim. Prod.* 40:351-358.

In 1972, revenue loss in the United States due to porcine MH was estimated to be at least a quarter of a billion dollars annually [Hall (1972) *Proc. of Pork Quality Symp.* (R.G. Cassens et al eds) pp. ix-xii].

20 Since the late 1960s and the mid 1970s, the incidence of MH in North American swine populations is reported to have decreased approximately twofold [Grandin (1980) *Internat. J. Studies in Anim. Product* 1:313-317). This decrease occurred as pig producers and meat packers

25 modified their swine breeding programs and swine- and pork-management practices in order to decrease the incidence of MH. This was achieved by reducing the use of heavily muscled swine as breeding stock, reducing pre-slaughter stress on market hogs and increasing carcass

30 chilling rates [Topel (1981) *Proc. Work Planning Meeting of FSE/DSD*, Ottawa, Agric. Can. 1-12].

Occurrence of MH is sporadic in all animal species except for swine, where, because of an association with heavy muscling, the disease has become endemic and in

35 some countries has reached epidemic proportions [Webb (1980) *Vet. Record* 106:410-412; Mitchell and Heffron (1982) *Ad. Food Res.* 18:167-230]. In swine, the

incidence of MH-susceptibility is breed and strain-dependent, ranging from less than 1% to greater than 90% of the herd. Increased prevalence of this disease, especially in European countries, is postulated to be a

5 reflection of genetic improvement programs based entirely on performance and production parameters such as depth of back fat, muscularity, and carcass yields.

For Dutch and German strains of Pietrain and Poland China, and German and Belgium strains of Landrace swine,

10 68 to 94% of individuals were found to be MHS homozygotes and heterozygotes. By contrast, less than 10% of swine in North American herds are found to have susceptibility to MH. Because these figures are based on a diagnostic test (halothane challenge test) with poor sensitivity,

15 they underestimate the incidence of pale, soft and exudative pork [Webb (1980;1981) *supra*]. Furthermore, swine and carcass management practices at slaughter affect the incidence and degree of pale, soft, exudative pork from MHS swine [Topel (1981) *supra*]. On the other

20 hand, these figures overestimate the occurrence (less than 1% in North America) of stress-induced MH-deaths, which may be largely eliminated by 'stress-free' management practices [Topel (1981) *supra*].

In Canadian swine, the prevalence of MH gene

25 homozygosity is up to 6% for Canadian Landrace and 1% for Yorkshire swine [D'allaire et al (1982) *Can. Vet. J.* 23: 168], the most important breeds. Based on recent data indicating that 1.9% of Ontario swine are homozygous for MH and a Mendelian inheritance pattern, the prevalence of

30 heterozygotes would be 24%. Administration of succinycholine in the halothane challenge test detects many heterozygotes.

The high fatality rate of MH, the recognition of its heritability, and its association with inferior quality

35 pork, have prompted the development of diagnostic tests for susceptibility to MH in swine. Halothane challenge testing is used extensively in the swine industry to

detect susceptibility.　Major limitations of this test,
however, include its low sensitivity [Nelson et al.
(1983) *J. Anesthesia Analgesia* 62:545-552; Seeler et al.
(1983) *supra*; Webb et al. (1986) *Anim. Prod.* 42:275-279]

5　and the high number of fatalities which may occur.　In a
typical test, two to three month old pigs are physically
restrained and forced to inhale 3 to 5　halothane in
oxygen through a face mask for several minutes.　Those
developing extensor muscle rigidity during the test are

10　diagnosed as MHS.　While the onset of MH signs is delayed
by prior tranquillization [McGrath et al. (1981) *Am. J.
Vet. Res.* 42:195-198], thermal, exertional,
pharmacological, or psychological stresses will speed its
onset [Van den Hende et al. (1976) *Brit. J. Anaesthesia*

15　48:821-829; Seeler et al. (1983) *supra*].

The caffeine [Kalow et al. (1970) *supra*] and
halothane [Ellis et al (19-1) *Brit. J. Anaesthesia* 43:
721-722] contracture tests are considered to be the most
definitive preanesthetic diagnostic tests for

20　MH-susceptibility [European Malignant Hyperpyrexia Group,
1984, *Brit. J. Anaesthesia* 57:983-990].　They are used
extensively in man and have been applied successfully in
swine [Gronert (1979) *Anaesthesia Analgesia* 58:367-371],
horses [Waldron-Mease and Rosenberg (1979) *Vet. Res.*

25　*Commun.* 3:45-50] and dogs [O'Brien et al. (1983) *supra*].

In veterinary medicine, contracture tests are seldom
used because of cost, the trauma involved and the need
for appropriate equipment and expertise.　In these tests,
muscle is excised, placed in oxygenated physiological

30　saline, and connected to a force-displacement strain
gauge.　Isometric tension of the muscle is transduced
into an electronic signal and recorded by a polygraph.
To demonstrate its viability, the muscle is made to
twitch continuously by applying electrical stimuli.

35　Caffeine or halothane is then added to the bathing
solution in increasing amounts.　Muscle from MHS
individuals is hypersensitive to the

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contracture-producing effects of these drugs. They increase the baseline tension of MHS muscle more, and at a lower concentration, than occurs for normal muscle [Britt, 1979, *Int. Anaesthesia Clin.* 17:63-96].

5   Diagnosis of MH-susceptibility may be facilitated by various indirect blood tests for MH, such as:

(1)   hap otyping swine for the MH-gene-linked marker loci for H [Rasmusen and Christian (1976) *Science* 191:947-948) and S (Rasmusen (1981)

10   *Anim. Bood Groups in Biochem. Genet.* 12:207-209] blood groups, erthyrocyte phosphohexose isomerase and 6-phosphogluconate dehydrogenase [Jorgensen et al. (1976) *Acta Vet. Scand.* 17:370-372], and serum protein

15   postalbumin-2 [Juneja et al. (1983) *Anim. Blood Groups in Biochem. Genet* 14:27-36];

(2)   erythrocyte osmotic fragility tests [King et al 1976, *Ann. Genet. Select. Anim.* 48:537-540];

(3)   hyperlactatemia and hemoconcentration following

20   intravenous injection of low dosages of halothane [Gregory and Wilkins (1984) *I. Sci. Food Agric.* 35:147-153];

(4)   post-exertional alterations in blood parameters, such as hyperlactatemia,

25   hemoconcentration, or elevated catecholamines [Ayling et al. (1985) *Brit. J. Anaesthesia* 57: 983-990; D'Allaire and DeRoth (1986) *Can. J. Vet. Res.* 50:78-83; Rand and O'Brien (1986) *Am. J. Vet. Res.* 190:1013-1014];

30   (5)   enzymatic [Schanus et al. (1981) *Prog. Clin. Biol. Res.* 55, 323-336] and thermochemiluminescent [Kiel and Erwin (1984) *Anal. Biochem.* 143:231-236) assays for antioxidant system deficiency;

35   (6)   electron microscopic detection of hypertrophied open canalicular systems and energy dispersive microanalytic detection of decreased surface

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membrane calcium in platelets [Basrur et al.
(1983) *Scanning Elect. Microsc.* 5:209-214];

(7)   abnormal halothane-induced purine nucleotide
profiles of platelet-rich plasma [Solomons and
5   Masson (1984) *Acta. Anaesth. Scand.* 27:
349-355];

(8)   abnormal halothane-induced increase in
cytosolic calcium of isolated lymphocytes [Klip
et al. (1986) *Biochem. Cell Biol.*
10   64:1181-1189].

These tests lack sensitivity, specificity,
reproducibility and/or have not been widely evaluated
[Britt (1985) *supra*; Lee et al (1985) *Anaesthesiol.*
63:311-315; O'Brien et al. (1985) *Am. J. Vet. Res.* 46:
15 1451-1456; Webb (1981) *supra*].

Tests which assay for the hypermetabolic and/or
degenerative changes occurring in 'triggered' MH muscle
include tests for ATP-depletion using *in vitro*
biochemical assays [Harrison et al. (1969) *Brit. J*
20 *Anaesthesia* 41:844-845] or *in vivo* 31-phosphorus nuclear
magnetic resonance spectroscopy [Roberts et al. (1983)
Anaesthesiol. 59:A230]; increased calcium efflux from
isolated mitochondria [Cheah and Cheah (1981) *Biochim.*
Biophys. Acta 634:40-49]; increased oxygen consumption
25 and muscle twitch response following local ischemia
[Jones et al. (1981) *Anesthesia Analgesia* 60:256-257] or
decreased calcium uptake by histologic sections [Allen et
al. (1980) *Anesthesiol.* 53:S251] or isolated sarcoplasmic
reticulum [O'Brien (1986) *Can. J. Vet Res.* 50:329-337].
30 These tests have not been standardized nor widely
evaluated. Furthermore, they may not be specific since
they are indirect, measuring changes occurring
secondarily to the underlying molecular defect.

A calcium-release sensitivity test performed on
35 isolated muscle sarcoplasmic reticulum [O'Brien (1986)
Can. J. Vet. Res. 50:318-329] is apparently a biochemical
correlate of the physiological caffeine and halothane

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contracture tests, although it may be one to two orders of magnitude more sensitive.

Recently direct analysis of the Ca^{2+} release channel protein has revealed differences that may correlate
5 directly with the disease:

(1) the Ca^{2+} release charnel of the sarcoplasmic reticulum (ryanodine receptor) from MHS pigs has a higher affinity for ryanodine binding and requires higher concentration of calcium to
10 inhibit ryanodine binding [Mickelson et al (1988) *J. Biol. Chem.* 263:9310-9315];

(2) patterns of tryptic digestion of the ryanodine receptor, as analyzed with a specific antibody, are altered in MHS swine [Knudson et al (1990)
15 *J. Biol. Chem.* 265:2421-2424];

(3) in single channel recordings, MHN Ca^{2+} release channels were inactivated by pCa less than 4, whereas MHS channels remained open for significantly longer times, demonstrating an
20 altered Ca^{2+} release channel inactivation [Fell et al (1990) *Biophys J.*50:471-475]

Calcium-release sensitivity tests and tests of the structure and function of the ryanodine receptor have not yet been used for medical or commercial purposes.
25 The calcium release channel of the sarcoplasmic reticulum (ryanodine receptor) is a large protein that spans the gap between the transverse tubule and the sarcoplasmic reticulum. The channel is activated by ATP, calcium, caffeine, and micro-molar ryanodine and
30 inhibited by ruthenium red, tetracaine, calmodulin, high Mg^{2+} and ryanodine (hence the name "ryanodine receptor") [Lai et al. (1988) *Nature* 331:315-319].

DNA encoding the human and rabbit skeletal muscle ryanodine receptors (RYR1) [Zorzato et al. (1990) *J.*
35 *Biol. Chem.* 265:2244-2256] and the cardiac muscle ryanodine receptor (RYR2) [Otsu et al (1990) *J. Biol. Chem.* 265:13472-13483] has been cloned and sequenced.

The deduced amino acid sequences of the RYR1 gene product comprise 5032 amino acids. The predicted protein structure suggests that the calcium release channel contains up to 12 transmembrane domains lying in the

5 C-terminal portion of the molecule. The remainder of the protein is predicted to constitute the "foot" portion which spans the gap between the transverse tubule and the sarcoplasmic reticulum. Potential binding sites for calcium, calmodulin, ATP and other modulators of calcium

10 channel function are also believed to be present in both RYR1 and RYR2 molecules between residues 2600 and 3000. Genetic linkage between the ryanodine receptor (RYR1) and MH genes has been determined in humans [MacLennan et al. (1990) *Nature* 343:359-361]. These observations strongly

15 support the view that the ryanodine receptor is involved in malignant hyperthermia and that a defect in the calcium release channel might be the basic defect in animals, including humans, with MH.

The gene responsible for halothane sensitivity (HAL)

20 has been found to segregate in pigs with a number of other genetic markers including S (S locus affecting expression of A-0 red blood antigens), Phi (glucose phosphate isomerase), H (H locus encoding blood group antigens), Po2 (postalbumin-2) and PgD (6-

25 phosphogluconate dehydrogenase) [Rasmussen and Christian (1976) *supra*; Rasmussen (1981) *supra*Jorgensen et al (1976) *supra*; Juneja et al (1983) *supra*]. It is, therefore, assumed that these genes are linked on pig chromosome 6p11-q21 [Horbitz et al. (1990) *Genomics*

30 8:243-248]. The human RYR1 gene has been localized to chromosome 19q13.1 and shown to be syntenic with these same genes [MacKenzie et al. (1990) *Am. J. Hum. Genet.* 46:1082-1089]. Linkage between the human RYR1 gene on chromosome 1gq13.1 and the MH gene has been established

35 in humans [MacLennan et al. (1990) *supra*].

We have discovered that the ryanodine receptor gene is the gene that is defective in porcine MH. This

invention relates to the isolation and sequence analyses of cDNAs encoding normal and mutant porcine skeletal muscle sarcoplasmic reticulum calcium release channel proteins (alternatively referred to as ryanodine

5　　receptors) and determination that a mutation in the RYR1 gene in MHS strains segregates with the MH phe otype in pigs, such as Yorkshire, Pietrain, Landrace and Duroc strains and, predictably in all other heavily muscled strains (eg. Poland China; hampshire). The discovery

10　that the porcine RYR1 gene is the gene responsible for MH and the discovery of diagnostic DNA probes from the porcine RYR1 gene forms the basis, according to an aspect of the invention, for a definitive DNA and/or antibody-based diagnostic test for MH.

15　　　　According to an aspect of the invention, substantially purified DNA sequence encoding porcine RYR1 and its normal and mutant functional equivalents is characterized by:

　　　　i)　the cDNAs encoding a protein having 5035 amino

20　　　acids and a molecular weight of approximately 564,000 daltons;

　　　　ii) the DNA having a length of approximately 15.3 kb; and

　　　　iii) isolated from chromosome 6 of pigs.

25　　　　According to another aspect of the invention, substantially purified normal and mutant porcine cDNAs have the sequences encoding the amino acid positions 1 to 5035 of Figure 2.

　　　　According to another aspect of the invention, DNA

30　probes include fragments of the DNA encoding the normal and mutant porcine ryanodine receptor protein.

　　　　According to a further aspect of the invention, the use of the DNA probes in mutational analysis determines if a porcine subject is homozygous or heterozygous for

35　susceptibility to malignant hyperthermia.

According to another aspect of the invention, the use of the DNA probes in a one-step PCR-based diagnosis of MHS mutation in pig genomic DNA is provided.

According to another aspect of the invention, at
5   least seventeen polymorphisms exist in normal and mutant porcine cDNAs as presented in Figure 2, such polymorphisms being useful in genetic analysis involving the ryanodine receptor.

According to another aspect of the invention,
10 substantially purified normal and mutant porcine ryanodine receptor proteins are free of any foreign porcine protein. The proteins have molecular weights of approximately 564,000 daltons and amino acid sequences as presented in Figure 2.

15 According to another aspect of the invention, substantially purified antibodies specific to protein sequences of the pig ryanodine receptor protein are disclosed. The antibodies may be either polyclonal antibodies or monoclonal antibodies. Such antibodies are
20 useful in immunological assays to determine if a pig is susceptible to malignant hyperthermia.

Aspects of the invention are demonstrated and described with respect to the drawings wherein:

Figure 1 is a schematic drawing showing the
25 restriction endonuclease map for the MH mutant and normal porcine ryanodine receptors;

Figure 2 is the nucleotide sequence (second row) and the deduced amino acid sequence (third row) of MH mutant porcine ryanodine receptor cDNA and the nucleotide
30 sequence (first row) of polymorphisms in the cDNA sequence and the normal amino acid arginine 615 of MHN (fourth row);

Figure 3 is a series of slot blot tests to detect the presence of normal and mutant alleles in various
35 identified breeds; and

Figure 4. (A) Nucleotide and deduced amino acid sequences of part of the exon in which the single

arginine 615 to cysteine mutation was located.   The
forward primer used in PCR amplification corresponded to
the sequence of nucleotides 1811 to 1834, which are
overlined, while the reverse primer was complementary to
5   nucleotides 1861 to 1884, which are also overlined.   The
alteration of the normal sequence GCGC1845 to the mutant
sequence GTGC1845 led to the deletion of a Hin PI
restriction endonuclease site in the mutant sequence.   A
second polymorphism (C in Yorkshire or G in Pietrain at
10   position 1878) is also noted; and
        Fig. 5.   (A) Detection of the arginine 615 to
cysteine mutation by oligonucleotide hybridization.
Autoradiographs show the binding of the two specific
oligonucleotide probes to the 74 bp PCR amplified product
15   identified in Fig. 1 from n/n Pietrain, N/N Yorkshire, or
heterozygous N/n individuals from Pietrain/Yorkshire
crosses (left), and of individuals from Yorkshire, Poland
China, Duroc and Landrace herds (right).   Oligonucleotide
probe 17-C detected the MHN DNA sequence and
20   oligonucleotide probe 15-T detected the MHS DNA sequence.
Both probes bind to heterozygote sequences.
        (B) Detection of the C to T mutation at
position 1843 by Hin PI restriction endonuclease cleavage
in the 74 bp PCR product from porcine genomic DNA (21).
25   Cleavage of the product generates 41 and 33 bp fragments.
Lanes 1 and 2, Hin PI digestion of one allele (Poland
China); lanes 3 and 4, digestion of both alleles (Duroc)
and lanes 5 and 6, lack of digestion (n/n Pietrain).
        (C) Detection of the A to G polymorphism at
30   position 4332 by Ban II restriction endonuclease cleavage
of a 160 bp PCR product from porcine genomic DNA (21).
Cleavage of the product generated 124 and 36 bp
fragments.   Lanes 1 and 2, Ban II digestion of one allele
(Duroc); lanes 3 and 4, lack of digestion (N/N
35   Yorkshire); and lanes 5 and 6, digestion of both alleles
(n/n Poland China).

(D) Detection of the G to A polymorphism at position 13878 by Rsa I restriction endonuclease cleavage of a 227 bp PCR product from porcine genomic DNA (21). Cleavage of the product generated 111 and 116 bp

5   fragments which move in the acrylamide gel as a single band. Lanes 1 and 2, digestion of one allele (N/n Yorkshire x Pietrain); lanes 3 and 4, digestion of both alleles (N/N Landrace); and lanes 5 and 6, lack of digestion (n/n Landrace).

10   Figure 6 - Nucleotide and deduced amino acid sequences of a part of the porcine ryanodine receptor gene in which the MH mutation is located. Capital letters indicate exon sequences, whereas small letters indicate intron sequences. The deduced amino acid

15   sequences are indicated for exon sequences. The locations of the primers used for PCR amplification of the 1.6 kb genomic sequence are doubly overlined. The locations of primers used for the amplification of the 659 bp fragment are singly overlined. The box indicates

20   the location and nature of nucleotide and amino acid alterations causative of MH. The constant *HgiAI* restriction endonuclease sites are underlined; the polymorphic *HgiAI* site is underlined with a hatched line.

         Figure 7 - Diagrammatic representation of digestion

25   patterns for the porcine genomic fragments obtained by PCR amplification. Thick horizontal lines represent exons, connected by thin horizontal lines representing introns. Horizontal arrows indicate location of forward and reverse primers. Vertical arrows indicate

30   polymorphic (C/T) sequences giving rise to polymorphic restriction endonuclease sites. Thin vertical lines indicate constant restriction endonuclease sites. The sizes of the fragments resulting from digestion with *HgiAI* are indicated above the line representing the

35   porcine gene.

         Figure 8 - Detection of the pig C1843 to T mutation by amplification and subsequent digestion of the

amplified product with *HgiAI*. A 659 bp PCR product was amplified from genomic DNA from N/N Yorkshire, n/n Pietrain, N/n Yorkshire x Pietrain and n/n British Landrace pigs. In each case, the first lane (-)

5 represents the PCR amplified product, while the second lane (+) represents the same product after digestion with *HgiAI*. Since the mutation of C1843 to T creates a *HgiAI* site, digestion of the N/N genotype with *HgiAI* generates 524 and 135 bp fragments from the constant *HgiAI* site and

10 358, 166, 135 bp fragment from the n/n genotype, through a combination of digestion of the constant and polymorphic *HgiAI* sites. Fragments of 524, 358, 166 and 135 bp fragments are generated in a N/n genotype from a combination of full digestion of the constant *HgiAI* site

15 and of one allele at the polymorphic *HgiAI* site, but no digestion of the other allele at the polymorphic *HgiAI* site. Note the differences in relative intensity of the four bands. In the case of BC69, 524, 358, 166 and 135 bp fragments are also generated from a pig diagnosed as

20 n/n by halothane challenge and haplotype testing. In this case, however, contamination of the sample with N/n DNA leads to retention of some of the 524 bp band. Contamination is detected by the abnormal intensities of the various bands (compare BC69 with N/n). With BC152, we

25 illustrate partial digestion of the 659 bp band. In this case a second, complete digestion led to diagnosis of this sample as n/n.

The restriction endonuclease map and sequencing strategy for the MH mutant and normal porcine RYR1 is

30 shown in Figure 1. More specifically, restriction endonuclease map and cloning, and sequencing strategies for cDNAs encoding for ryanodine receptor are from MH (Pietrain strains) and normal (Yorkshire strain) skeletal muscles. A series of linear clones were isolated from a

35 Pietrain strain skeletal muscle cDNA library in lambda gt10. These clones are illustrated in the upper half of Figure 1. A second series of cDNA clones was isolated

from a Yorkshire strain skeletal muscle cDNA library in
lambda ZAP. These clones are illustrated in the lower
half of Figure 1. The long central filled bar
illustrates the size of the coding sequence, while the
5   open bars at either end represent the sizes of 5' and 3'
untranslated sequences. The locations of 6 basepair
restriction endonuclese sites are noted. Short
intermediate bars indicate the sizes and locations of
cDNA clones isolated and sequenced from MH (upper) and
10   normal (lower) cDNA libraries. For the MHS sequence
(upper), arrows indicate the extent and direction of
most, but not all, of the cDNA sequencing that was
carried out. Sequencing in both directions was carried
out for 93.3% of the sequence following subcloning of
15   portions of the larger clones (not indicated).
Sequencing in one direction of four independent clones
was carried out for nucleotides-129 to 831 and of five
independent clones for nucleotides 12502-12567. For the
MHN sequence (lower) only the overlaps and duplications
20   indicated were sequenced more than once. Short hatched
bars indicate two regions where cDNAs were isolated and
sequenced from polymerase chain reactions (PCR) [Saiki,
R.K. et al *Science* 239:487-491 (1988)]. The lower thin
bar is a marker indicating the size of the cDNA in
25   kilobase pairs.

To obtain pig cDNA libraries, mRNAs were isolated
from frozen muscle of the homozygous MH pig (Pietrain
strain) and the homozygous normal pig (Yorkshire strain)
[MacLennan and DeLeon (1983) *Methods Enzymol.*]. cDNA
30   libraries were constructed using standard protocols
supplied by the manufacturers of standard cDNA cloning
kits.

The normal and mutant pig cDNA clones, it was
discovered, could be obtained by screening the cDNA
35   libraries with human RYR1 cDNA fragments [Zorzato et al.
(1990) *supra*] to obtain clones covering the entire
sequence. The nucleotide sequence for human cDNA is

published [Zorzato et al. (1990) *supra*] and hence is
readily available to the public. It is understood by
those skilled in the art, that any library prepared from
porcine skeletal muscle according to established methods
5　should be representative of the RNA species present in
the tissue and should, therefore, be the source of
porcine ryanodine receptor clones.

Pigs predisposed to MH have functional
excitation/contraction coupling in the absence of
10　triggering agents, although the Ca^{2+} release channel
appears to have a hypersensitive gating mechanism
[O'Brien, J. P., *J. Mol. Cell. Biochem.* 93:53-59
(1990)]. Accordingly, it was unlikely that the RYR1
gene, potentially causative of MH, would be altered by
15　major deletions or premature stop codons. In order to
test the postulate that a point mutation or short
deletion would exist in the RYR1 gene that would be
causal of porcine MH, we sequenced full length ryanodine
receptor cDNA fragments from both an MH Pietrain pig and
20　an MH normal (MHN) Yorkshire pig as already noted in
Figure 1. Sequencing of the Pietrain ryanodine receptor
cDNA demonstrated the absence of a deletion or an
internal stop codon. A comparison of the porcine amino
acid sequence with published amino acid sequences for
25　human [Zorzato et al *J. Biol. Chem* 265:2244-2256(1990)]
and rabbit [Zorzato et al, *supra*; Takeshima et al *Nature*,
339:439-445 (1990)] showed about 180 differences between
rabbit and pig, between human and pig, or between human
and rabbit, and about 90 unique amino acid differences
30　for each of the three sequences.

Figure 2 is the nucleotide sequence (second row) and
deduced amino acid sequence (third row) of the MH mutant
(Pietrain strain) porcine ryanodine receptor cDNA. Fig.
2 is also the nucleotide sequence (first row) and deduced
35　amino acid sequence (4th row) of the normal (Yorkshire)
porcine ryanodine receptor cDNA (only differences from
Pietrain are recorded for the Yorkshire sequence). The

nucleotide and amino acid designations are in keeping with international convention and are well understood by those skilled in the art. Specifically, the nucleotide and deduced amino acid sequences of cDNAs encoding MH and

5   normal porcine skeletal muscle ryanodine receptors are listed. Nucleotides are numbered positively in the 5' to 3' orientation beginning with residue 1 of the ATG codon for the initiator methionine and negatively for the 5' untranslated region, beginning from the 5' side of

10   residue 1. Amino acids are numbered positively beginning at the initiator methionine codon. Each group of lines has 4 components: i) the normal nucleotide sequence where it differs from the MH sequence (17 polymorphisms are noted); ii) the nucleotide sequence of the MHS

15   ryanodine receptor cDNA; iii) the deduced amino and sequence of the MHS cDNA; iv) the amino acid sequence of the normal ryanodine receptor where it differs from MHS. The single amino acid substitution, cysteine 615 (MHS) for arginine 615 (MHN), is boxed.

20   In order to detect a point mutation in the Pietrain RYR1 gene, we sequenced a full length cDNA from the MHN Yorkshire breed for comparative purposes. We detected the already noted 17 polymorphisms between the two breeds which are identified in Figure 2. Only one of the

25   polymorphisms, replacement of C1843 in the cDNA from the MHN breed with T1843 in the cDNA from the MHS breed led to an alteration in the amino acid sequence from arginine 615 in the MHN animal to cysteine 615 in the MHS animal.

      The deduced amino acid sequence of the open reading

30   frame is numbered from 1 to 5035. The 3' untranslated region, beginning after the TGA termination codon, is 144 bp long. A canonical polyadenylation signal AATAAA [Proudfoot and Brownlee (1976) *Nature* 263:211-214] is found 16 bases upstream of the polyadenylation site and

35   this is followed closely by a TG-rich sequence, characteristic of sequences between the polyadenylation signal and the polyadenylation site [McLauchlan et al

(1985) *Nucleic Acids Res.*, 13:1347-1368]. The initiator
methionine is 15105 bp upstream of the termination codon.
The initiator methionine codon is present in the longer
sequence ACATCATGG which closely resembles the consensus

5   initiation sequence, AC(A)TCATGG [Kozak (1984) *Nature*
308:241-246]. The normal and mutant porcine cDNA
sequences of Figure 2 encode proteins of 5035 amino acids
with predicted molecular weights of approximately
564,743.

10   It is understood that the term substantially pure as
used herein means that the isolated and purified DNA or
protein is free of any foreign animal DNA or protein. It
is also understood that, with reference to disclosed and
claimed DNA or protein sequences, various functional

15   equivalents exist which are due to substitutions in
variable regions of the DNA sequence or protein which do
not affect the essential function of the DNA sequence or
protein sequence.
           Proof that the cDNAs coded for normal and mutant

20   porcine ryanodine receptor proteins was based on their
colinearity and high degree of sequence identity (greater
than 95%) with published sequences of rabbit and human
[Zorzato et al. (1990) *supra*] and pig [Harbitz et al
(1990) *supra*] RYR1 cDNAs. Proof that the gene giving

25   rise to the cDNA is located on pig chromosome 6 is
provided by a comparison of this sequence with a partial
sequence localized to pig chromosome 6 by in situ
hybridization [Harbitz et al (1990) *supra*).
           Although 17 nucleotide sequence differences are

30   noted between normal and pig sequences, only one, the
alteration of C to T at position 1843, leads to a change
in amino acid sequence from arginine 615 to cysteine 615.
These nucleotide and resulting amino acid sequence
changes in the RYR1 gene are causative of the

35   predisposition to MH.
           The porcine ryanodine receptor sequence is typical
of other RYR1 gene products. The deduced amino acid

●

●

19

sequence is slightly longer than the human and rabbit
sequences (5035 vs. 5032 amino acids), but its molecular
weight of 564,743 is similar to that of both skeletal
[Takeshima et al (1990) *supra*; O'Brien J.P. et al, *Can.*

5   *J. Vet. Res.* 54:8392 (1990)] and cardiac [Otsu et al,
(1990) *supra*, Furuichi et al *Nature* 342:32-38 (1989)]
forms. The short 3'untranslated sequence of 144 bases
has a canonical polyadenylation signal 16 bp upstream of
the polyadenylation site. Analysis of the predicted

10   structure of the molecule shows the same features
recognized in earlier analyses of other ryanodine
receptors [Zorzato et al (1990) *supra*; O'Brien et al
(1990) *supra*; Otsu et al *J. Biol Chem.* 265:13472-13483
(1990)].

15   Arginine 615 lies in the NH2-terminal 1/8 of the Ca^{2+}
release channel protein and is part of a long hydrophilic
cytoplasmic domain which includes at least the first 4000
residues in the molecule. Mignery and Sudhof [*supra*]
have demonstrated that the InsP3 binding region lies in

20   the NH_2-terminal quarter of the $InsP_3$ receptor. The $InsP_3$
and ryanodine receptors are homologous [Furuichi et al
supra] and there is partial sequence identity between the
$InsP_3$ binding region of the $InsP_3$ receptor and the region
of the ryanodine receptor surrounding arginine 615. Thus

25   it is reasonable to assume that arginine 615, like the
homologous residue in the $InsP_3$ receptor, lies in a region
of the ryanodine receptor that is concerned with the
binding of regulators of Ca^{2+} release channel gating.

        Analysis of the sequence surrounding arginine 615

30   suggests that it lies within a beta strand domain
comprised roughly of amino acids 520 to 830 [Zorzato et
al (1990) *supra*]. The relatively hydrophobic sequence
immediately preceding arginine 615 contains an
interesting repeat motif of 2 hydrophobic, 1 hydrophilic

35   and 2 hydrophobic residues (IVNLL522; LASLI530; LVSKL551;
ILEVL564; VLNII578; IISLL590; LLDVL603; VCNGV612). The
sequence after arginine 615 contains at least 9 predicted

beta strands which end at the beginning of a tandem
repeat structure [Zorzato et al (1990) *supra*] between
residues 842 and 1015.

5   Alteration of an Arg-Ser bond in the MHN sequence to
a Cys-Ser bond in the MHS sequence deletes a tryptic
cleavage site in the MHS protei. Knudson et al [(1990)
supra] have reported an alterat.on in the early tryptic
cleavage pattern of the ryanodine receptor protein in
which a 99 kDa tryptic peptide in MHS pigs replaces an 86

10   kDa tryptic peptide in MHN pigs. This alteration would
be consistent with the deletion of a tryptic cleavage
site in the abnormal 99 kDa fragment which would give
rise to 86 and 13 kDa tryptic fragments in the normal
sequence. The Arg to Cys mutation that we have detected

15   is, according to our discovery, responsible for the
altered tryptic cleavage pattern observed by Knudson et
al [(1990) *supra*]. The fact that the tryptic cleavage
pattern alteration is so readily evident at such early
times of digestion suggests that the Arg-Ser 616 bond is

20   exposed to trypsin. Arginine 615 is therefore located
on the surface of the Ca^{2+} release channel, even though
this was not predicted by Marks et al [*J. Biol. Chem.*
1990) <u>265</u>:13143-13149].

        It is not yet clear how the mutation of Arginine 615

25   causes MH susceptibility. Studies of the physiological
function of the MHS Ca^{2+} release channel show that it is
opened by Ca^{2+}, ATP and caffeine at concentrations several
fold lower than those required for the MHN channel and
that its inhibition by Mg^{2+} is dampened [O'Brien, P.J.

30   (1987) *supra*, O'Brien, P.J. (1986) *supra* and O'Brien,
P.J. (1990) *supra*]. Thus at comparable concentration of
these triggering agents, Ca^{2+} release rates are higher for
the sarcoplasmic reticulum from MHS than from MHN
individuals [Mickelson et al *supra*]. Moreover, high

35   affinity ryanodine binding to the Ca^{2+} release channel,
which occurs in the open channel configuration [Fleischer
et al, *Proc. Natl. Acad. Sci. USA* (1985) <u>82</u>:7256-7159],

has an altered Ca^{2+} dependency, presumably related to the
extent of Ca^{2+} channel opening. Altered Ca^{2+} dependence
of Ca^{2+} release channel inactivation (closing) has also
been demonstrated [Gill et al (1990) *Biophys. J.* 50:471-
5   475]. These observations are consistent with the
postulate that the MH mutation leads to channel gating
which is hypersensitive to triggering agents [O'Brien,
P.J. (1987) *supra*, O'Brien, P.J. (1986), O'Brien, P.J.
(1990) *supra* and O'Brien et al (1990) *supra*]. This
10   postulate is further supported by analogy with a
homologous Ca^{2+} release channel. The inositol 1, 4, 5
trisphosphate (IP_3) receptor [G.A. Mignery, and T.C.
Sudhof, EMBO J. 9, 3893 (1990)] has demonstrated that IP_3
binds to the to the NH_2-terminal quarter of the IP_3
15   receptor in a region homologous between the two
receptors. There is 31% sequence identity between the 39
amino acids surrounding arginine 615. This homologous
region is a strong candidate site for ligand binding in
both receptors.
20   It is not fully understood why the mutation leads to
the sustained channel opening, characteristic of the
disease, when the MHS individual is exposed to
inhalational anaesthetics or other MH triggering agents
or to stress. A plausible explanation, which is
25   understood to be non-limiting to this invention, is that
MH triggering agents such as anaesthetics or
stress-induced neuromuscular and endocine stimulation
directly alter the intracellular concentrations of
physiological channel gating agents such as Ca^{2+}, ATP,
30   calmodulin or Mg^{2+}[27] to the point where they will trigger
opening of the hypersensitive MHS channel, but not of the
MHN channel. Once opened, the MHS channel will also
respond poorly to Ca^{2+}- and Mg^{2+}- induced inactivation
[Gill et al (1990) *supra*], thereby remaining open to
35   induce muscle contracture, hypermetabolism and,
ultimately, fever.

The relationship between the MH mutation in swine and the MH mutation in humans is also not fully understood.   In all respects except inheritance, the pig and human diseases are believed to be analogous [Britt

5   (1987) *supra*].   MH susceptibility has been proposed to be inherited in a dominant pattern in people and in ı recessive pattern in swine [Britt (1987) *supra* ar.d Denborough et al (1960) *supra*].   This postulate is probably invalid since the porcine halothane challenge

10   test used in swine genetic studies has been designed to detect homozygotes and to spare the lives of heterozygotes.   By contrast, the conditions for detection of MH in humans either *in vivo* inhalation of high levels of halothane for prolonged periods in the presence of

15   succinyl choline; *oor in vitro* halothane/caffeine contracture tests [Britt (1987) *supra*] detects human heterozygotes (homozygous MH humans are rare). Inheritance is, therefore, described as dominant.   A comparable test can detect pigs heterozygous for the MH

20   mutation also, thereby leading to a change in the designation of the swine inheritance pattern from recessive to dominant, the same designation as for humans.   If MH susceptibility is similar in pigs and humans, it is likely that mutations in the same

25   structural and functional domain of the RYRI gene will be causative of the disease in both species.

            The site of the MHS mutation, as exposed on the surface, is a potential target for the binding of site directed, differential antibodies that can be used in the

30   development of an antibody-based diagnostic assay for MH that could be carried out on the protein from isolated muscle fractions or in tissue sections.

            In order to provide evidence that the amino acid sequence alternation is causative of MH, we carried out

35   diagnosis of the association of the T1843 mutation with the MHS phenotype in a variety of pigs of deferring MH susceptibility status.   Blood or muscle samples were

isolated from 149 individual pigs at the University of
Guelph for DNA based diagnosis at the University of
Toronto. Tissue samples were numbered, but not otherwise
identified for the Toronto study. Subsequently, 37 of

5   these same tissue samples were provided for an additional
blind repeat test. Of the 149 samples, 48 homozygous MHS
Pietrain, 9 homozygous MHN Yorkshire, 2 heterozygous
Yorkshire and 20 heterozygotes resulting from MHS
Pietrain by MHN Yorkshire crosses had been subjected to

10   diagnosis of MH susceptibility by the halothane challenge
[O'Brien et al (1990) *supra*], the caffeine/halothane
contracture test [O'Brien et al (1990) *supra*] or the
Ca^{2+}release channel activity test [O'Brien et al, *Can. J.
Vet. Res* 50:318-328 (1986)], as well as by full knowledge

15   of the genetic background of the parental animals.

Genomic DNA was isolated from these animals and a 75
bp sequence, lying within a single exon, was amplified by
the polymerase chain reaction (PCR) [Saiki et al *Science*
239:487-491 (1988)]. The forward primer used in PCR

20   amplification corresponded to nucleotides 1811 to 1834 in
Figure 2. The reverse primer was complementary to
nucleotides 1861 to 1884 in Figure 2.

A 17-mer oligonucleotide sequence of the composition
5'-TGGCCGTGCGCTCCAAC-3' (probe 17-C) and a 15-mer

25   oligonucleotide of the composition 5'-GGCCGTGTGCTCCAA-3'
(probe 15-T) were synthesized, end labelled with ^{32}P and
used as probes [Maniatis et al *Molecular Cloning: A
Laboratory Manual* (Cold Spring Harbor Laboratory, New
York, 1982)] to detect the presence of normal and mutant

30   alleles in the various breeds.

These two oligonucleotides were used as differential
hybridization probes to detect the presence of C and T
alleles in the various breeds as shown in Figure 5A. In
view of the mutation deleting a Hin P1 site, as

35   specifically noted in Figure 4, reliance of this loss of
site was used in the same PCR product as a restriction
enzyme test, the results of which are shown in Figure 5B.

Figure 3 is an autoradiograph illustrating the diagnosis of homozygous MH susceptible, homozygous normal and heterozygous MH susceptible/normal alleles in Pietrain, Landrace, Duroc and Yorkshire pigs. These
5   DNAs are slot blotted [Tlsty et al (1982) in "Gene amplification" (R.C. Schimke ed.) *CSH Laboratory Press* pp. 231-238] onto BA85 nitrocellulose paper as described by the manufacturer (Schleicher and Schuell) baked for 2 hours at 80°, prehybridized for 2 hours and hybridized
10   for 4 hours with one or the other of two ^{32}P-labelled oligonucleotide probes in a solution containing 6 x SSC 0.1% sodium dodecyl sulfate and 10 x Denharts solution. The first probe, of the composition (probe 17-C), binds selectively to the normal sequence at 61° in the presence
15   of 6 x SSC and 0.1% SDS while the second, of the composition (probe 15-T) binds selectively to the corresponding MH susceptible sequence at 55° in the same buffer. Heterozygous DNAs will bind both probes at the two different temperatures. In Figure 3, we illustrate
20   that these two probes will differentiate homozygous MHS, homozygous MHN and heterozygous genotypes in Pietrain, Yorkshire and Yorkshire/Pietrain crosses. Of the 149 animals in which DNA based diagnosis was compared with standard methods of diagnosis of MH susceptibility, there
25   was high level of agreement between the two methods (See Table 1). The study therefore proves that the T1843 for C1843 mutation in the DNA sequence, leading to a cysteine 615 for arginine 615 substitution in the amino acid sequence, is correlated with MH susceptibility in
30   Pietrain and Yorkshire swine. The conclusion that this mutation is causative of MH susceptibility is therefore fully justified.

Study of the correlation between phenotypic and genotypic diagnosis is complicated by the lack of a well
35   defined phenotypic test for the N/n (heterozygous MH) genotype and by the fact that a low percentage of animals reacting to the commonly used halothane challenge test

are of the N/n genotype while a low percentage of those
that do not react are of the n/n genotype (homozygous MH)
[P.J. O,Brien, Vet. Res. Commun. 11:527 (1987); P.J.
O'Brien, A. Klip, B.A. Britt, B.I. Kalow, Can. J. Vet.

5   Res. 54:83 (1990); P.J. O'Brien et al., Am. J. Vet. Res.
46:1451 (1985)]. The T/T allelic pair was found at
nucleotide 1843 in each of 48 pietrain animals from an
inbred herd. All breeding stock used in development of
this herd had been shown to be n/n by testing of progeny

10   using the halothane challenge test or the
halothane/caffeine contracture test [P.J. O,Brien, A.
Klip, B.A. Britt, B.I. Kalow, Can. J. Vet. Res. 54:83
(1990); P.J. O,Brien et al., Am. J. Vet. Res. 46:1451
(1985]. Of these 48, 11 were diagnosed in this study as

15   n/n in a halothane/caffeine contracture test and 6 in the
test for hypersensitive gating of the Ca^{2+} release channel
(S.T. Ohnishi, S. Taylor. G.A. Gronert, FEBS Lett.
161:103 (1983); P.J. O'Brien, Mol. Cell. Biochem. 93:53
(1990)]. The C/C allelic pair was found in 36 of 43 and

20   the C/T allelic pair in 7 of 43 Yorkshire animals from a
closed herd, also inbred for 10 years for halothane
resistance, but originating in commercial stock
in which 0.78% have been found to be positive for the
halothane challenge test Pietrain and Yorkshire swine

25   were obtained from closed herds with inbreeding programs
controlled for more than 10 years. Selection of Pietrain
breeders was based on production of offspring positive
for either the halothane challenge test or the halothane
and caffeine contracture test, the reference tests for

30   diagnosis of MH susceptibility [P.J. O'Brien, Vet. Res.
Commun. 11:527 (1987); B.A. Britt, in Malignant
Hyperthermia, B.A. Britt, ed. (Martinus Nijhoff, Boston,
1987), pp. 11-42; P.J. O'Brien, A. Klip, B.A. Britt, B.I.
Kalow, Can. J. Vet. Res. 54:83 (1990)]. Selection of

35   Yorkshire breeders was based on negative halothane
challenge tests and production of offspring negative for
this test. In the halothane and caffeine contracture

tests, swine were considered MH-susceptible if a 1 g increase in tension (CSC) occurred with less than 4 mM caffeine or if more than 0.5 g tension developed with 5% halothane; normal if CSC was greater than 7 mM and

5 halothane produced less than 0.2 g increase in tension; and heterozygous if CSC was between 5 and 6 [P.J. O,Brien, B.I. Kalow, N. Ali, L.A. Lassaline, J.H. Lumsden, J.H. Am. J. Vet. Res. **51**:1038 (1990)]. To confirm the presence of a functional defect in the

10 ryanodine receptor in MHS swine, microassays for hypersensitive gating of the Ca^{2+} release channel [S.T. Ohnishi, S. Taylor, G.A, Gronert, FEBS Lett. **161**:103 (1983); P.J. O'Brien, Mol. Cell. Biochem. **93**:53 (1990);] were performed on isolated skeletal muscle sarcoplasmic

15 reticulum. Half-maximal Ca-release occurred at less than 2 mM caffeine for all Pietrains and at greater than 7 mM caffeine for all Yorkshires. The estimate of the allele frequency of 8.8% for 24 Duroc swine unrelated to known reactor swine is based on the assumption that it is

20 similar to that of Ontario Yorkshire swine [D.C. Seeler, W.M. McDonell, P.K. Basrur, Can. J. Comp. Med. **47**. 284 (1983). This estimate was modified to 31.3%, however, for a group of 8 Durocs, 6 of which were the offspring of 2 which had produced both reactor and non-reactor

25 offspring and were, therefore, heterozygotes. The combined T allele frequency for 32 Durocs, therefore, was estimated to be 14.4%]. Of the animals studied, 9 were diagnosed as N/N by the halothane/ caffeine contracture test and found to be C/C in the DNA based test. Two had

30 intermediate halothane/caffeine contracture test results and were tentatively diagnosed as N/n and shown to be C/T in the DNA based test. The finding of a T allele frequency of 8.1% in this inbred herd was correlated with the earlier finding of 8.8% for the allele in the

35 Yorkshire population from which the herd was derived and 9.0% based on our present halothane/caffeine contracture test results for 11 animals [Pietrain and Yorkshire swine

were obtained from closed herds with inbreeding programs
controlled for more than 10 years. Selection of Pietrain
breeders was based on production of offspring positive
for either the halothane challenge test or the halothane
5   and caffeine contracture test, the reference tests for
diagnosis of MH susceptibility [P.J. O'Brien, Vet. Res.
Commun. 11:527 (1987); J.A. Britt, in Malignant
Hyperthermia, B.A. Britt, ed. (Martinus Nijhoff, Boston,
(1987), pp. 11-42; P.J. O'Brien, A. Klip, B.A. Britt,
10   B.I. Kalow, Can. J. Vet. Res. 54:83 (1990)]. Selection
of Yorkshire breeders was based on negative halothane
challenge tests and production of offspring negative for
this test. In the halothane and caffeine contracture
tests, swine were considered MH-susceptible if a 1 g
15   increase in tension (CSC) occurred with less than 4 mM
caffeine or if more than 0.5 g tension developed with 5%
halothane; normal if CSC was greater than 7 mM and
halothane produced less than 0.2 g increase in tension;
and heterozygous if CSC was between 5 and 6 [P.J.
20   O'Brien, B.I. Kalow, N. Ali, L.A. Lassaline, J.H.
Lumsden, J.H. Am. J. Vet. Res. 51:1038 (1990)]. To
confirm the presence of a functional defect in the
ryanodine receptor in MHS swine, microassays for
hypersensitive gating of the Ca^{2+} release channel [S.T.
25   Ohnishi, S. Taylor. G.A. Gronert, FEBS Lett. 161:103
(1983); P.J. O,Brien, Mol. Celle Biochem. 93:53 (1990);]
were performed on isolated skeletal muscle sarcoplasmic
reticulum. Half-maximal Ca-release occurred at less than
2 mM caffeine for all Pietrains and at greater than 7 mM
30   caffeine for all Yorkshires. The estimate of the allele
frequency of 8.8% for 24 Duroc swine unrelated to known
reactor swine is based on the assumption that it is
similar to that of Ontario Yorkshire swine [D.C. Seeler,
W.M. McDonell, P.K. Basrur, Can. J. Comp. Med. 47. 284
35   (1983). This estimate was modified to 31.3%, however,
for a group of 8 Durocs, 6 of which were the offspring of
2 which had produced both reactor and non-reactor

offspring and were, therefore, heterozygotes. The
combined T allele frequency for 32 Durocs, therefore, was
estimated to be 14.4%]. Of 14 animals derived from
crosses of Pietrain X Yorkshire, all had the C/T
5   genotype, as did 5 proven halothane resistant Landrace
animals resulting from the mating of animals from inbred
halothane resistant and halothane sensitive lines. A
single Poland China pig, diagnosed as n/n by both
breeding and halothane/caffeine contracture testing, had
10   the T/T phenotype. The DNA based test was also applied
to 71 animals from commercial herds in which a low
frequency of halothane susceptible animals was known to
exist, but in which the N/n and N/N animals were not
distinguished phenotypically. It was found that 11 C/T
15   and 21 C/C genotypes among 32 Durocs, resulted in a
frequency for the T allele of 17.2%, where the predicted
frequency was 14.4% and a T allele frequency of 9.1% in
11 Poland China where the T allele frequency was
predicted to be comparable to that in Yorkshires -
20   (8.8%). No T alleles were found in 20 Hampshire pigs,
correlating with the fact that MH Hampshire have never
been reported in Canada. Eight Landrace halothane
reactors were analyzed where N/n and n/n could not be
distinguished phenotypically and 5 C/T and 3 T/T
25   genotypes were found. Estimates of allele frequency
based on phenotypic analyses were highly correlated with
genotypic analysis (P<0.0001).

TABLE I

(a) Agreement Between Diagnosis of MH Susceptibility by Caffeine and Halothane Hypersensitivity Tests and Diagnosis by DNA-based Test

Diagnosis		Swine tested	Tests done	P value
N/N	versus	9	12	10^{-12}
N/n or /n/		77	107	
n/n	versus	48	68	10^{-4}
N/N or N/n		93	104	
N/n	versus	15	23	10^{-7}
N/N or n/n		57	81	

P values indicate the statistical significance for agreement of all diagnoses as determined by Cochran's Q-test for agreement of categorial data.
N/N = homozygous normal phenotype
n/n = homozygous MH phenotype
N/n = heterozygous MH phenotype

(b) Correlation of estimates of MH gene frequency in different herds by different hypersensitivity tests and by the DNA-based test

Herd	Swine Tested	MH Gene Frequency hypersensitivity test	Estimate DNA-probe test
Inbred Pietrain (P)	48	1.00	1.00
Normal Yorkshires	9	0.00	0.00
Heterozygous Yorkshires (Y)	2	0.50	0.50
P x Y crossbreds	14	0.50	0.50
Y with no homozygous MHS pigs	43[a]	0.09	0.08
Halothane reactive MHS Landrace	8	0.50 - 1.0	0.68

Halothane resistant MHS Landrace	5	0.50	0.50
Duroc with no homozygous MHS but producing homozygous MHS pigs	31	0.50 - 0.15	0.10

The P value for correlation of estimates of MH gene frequency is 10^2 as determined by David's r-test for correlation.

• Estimate of gene frequency by caffeine hypersensitivity tests is based on 11 animals.

We extended the study to a herd of Yorkshires (32 animals), believed to be free of MH, and to representatives of herds of Duroc (31 animals) and Landrace (19 animals) breeds in which MHS susceptible animals had been detected by the halothane challenge test. DNA based diagnosis of the individuals from all of these herds revealed the presence of heterozygotes for the MH mutation (Figure 2). DNA based diagnosis of these animals was not verified by other specific symptomatic tests. The study clearly illustrates that diagnosis by a DNA based test is feasible for Pietrain, Yorkshire, Duroc and Landrace breeds, all of which are characterized as lean, heavily muscled pigs.

The fact that the probes are useful for the diagnosis of MH in four major breeds of lean, heavily muscled pigs strongly suggests that the MH gene in these strains has a single common origin and that it has been introduced into these breeds from a common source.

To see if the mutation is associated with a specific haplotype in all breeds we analysed the cosegregation of the mutation at nucleotide 1843 with a polymorphism at nucleotide residue 4332 (Table II) which alters a Ban II site within a 160 bp exon in the ryanodine receptor gene (Fig. 5C) and of a polymorphism at nucleotide 13878 (Table II) which alters an Rsa I site within a 261 bp exon (Fig. 5D). By analogy with the human gene (M. Phillips, J. Fujii and D.H. MacLennan, unpublished), the

Ban II site is about 35 kbp and the Rsa I site 145 kbp downstream of the Hin Pl site.

The Hin Pl cleavage site, incorporating nucleotide 1843, is absent in inbred Pietrain DNA, the Ban II cleavage site is present and the Rsa I cleavage site is absent, so that the MH Pietran haplotype is Hin Pl⁻ Ban II+ Rsa I⁻. Data presented in Table III show that all diagnosed n/n individuals from Pietrain, Landrace and Poland China breed carried only the Hin Pl⁻ Ban II⁺ Rsa I⁻ haplotype. The Ban II$^{-/-}$ and Rsa I$^{+/+}$ allelic pairs were excluded from all Him Pl$^{+/-}$ individuals, tentatively identified as N/n, so that all Hin Pl$^{+/-}$ individuals in Yorkshire, Duroc, Landrace and Poland China breeds contained a potential Hin Pl⁻ Ban II⁺ Rsa I⁻ haplotype. In Him Pl$^{+/+}$ individuals, tentatively identified an N/N in each of the six breeds, the Hin Pl$^{+/+}$ genotype coexisted with several combinations of Ban II and Rsa I polymorphisms, including Ban II$^{+/+}$ Rsa I$^{-/-}$, the genotype that was found for these two polymorphisms in all Hin Pl^{--} animals, and the Ban II$^{-/-}$ Rsa I$^{+/+}$ genotype, excluded from the Hin Pl$^{+/-}$ genotype. On the basis of these results, a common origin for the chromosome containing the T allele and, by association, a common ancestry for all the MH animals in all five breeds, as indicated.

Knowledge of this molecular alteration associated with MH and with specific RYR1 haplotype has allowed us to develop a simple, accurate, and non-invasive test for the altered RYR1 gene that will make it possible to eliminate this gene from each of these breeds if that is desired. The high frequency of MH susceptibility in Pietrain, Yorkshire, Poland China, Duroc and Landrace breeds of swine, however, may result from the widespread selection for leanness and muscularity in breeding stock. A clear demonstration of an association between a defective RYR1 gene and lean body mass might make it advantageous to reintroduce the defective RYRI gene selectively into breeding stock (1)

to yield F1 generation N/n market animals which might all
have superior lean body mass (1) and acceptable meat
quality (23).

TABLE II

Nucleotide Amino Acid	Yorkshire	Pietrain
276 (92)	TCC (Ser)	TCA (Ser)
1843 (615)	CGC (Arg)	TGC (Cys)
1878 (626)	CTG (Leu)	CTC (Leu)
3942 (1314)	GGT (Gly)	GGC (Gly)
4332 (1444)	GAA (Glu)	GAG (Glu)
4365 (1455)	CCC (Pro)	CCT (Pro)
6738 (2246)	CAA (Gln)	CAG (Gln)
7563 (2521)	CAT (His)	CAC (His)
7809 (2603)	GTC (Val)	GTT (Val)
8811 (2937)	GCG (Ala)	GCA (Ala)
9063 (3021)	ACT (Thr)	ACA (Thr)
9456 (3152)	CAG (Gln)	CAA (Gln)
9471 (3157)	GTC (Val)	GTA (Val)
9822 (3274)	ACG (Thr)	ACA (Thr)
9982 (3328)	CTG (Leu)	TTG (Leu)
11121 (3707)	ACT (Thr)	ACC (Thr)
12171 (4057)	TTT (Phe)	TTC (Phe)
13878 (4626)	GTG (Val)	GTA (Val)

TABLE III

Haplotype/genotype			Breed						
HinPI	BanII	RsaI	Pietrain	Yorkshire	PolandChina	Duroc	Landrace	Hampshire	Total
T/T									
-/-	+/-	+/-	18/18	6/9	5/6	8/10	4/11	-	23/36
-/-	+/-	+/-	-	-	1/6	-	-	-	1/36
C/T									
-/-	-/+	+/+	-	1/9	-	1/10	1/11	-	5/36
-/-	+/+	+/+	-	2/9	-	1/10	6/11	-	9/36
C/C									
+/+	+/-	+/-	-	-	1/4	3/8	-	5/20	9/45
+/+	+/+	+/+	-	2/12	-	-	-	-	2/45
+/+	+/+	+/+	-	4/12	3/4	4/8	1/1	2/20	14/45
+/+	+/+	-/-	-	-	-	1/8	-	-	1/45
+/+	-/-	-/-	-	6/12	-	-	-	13/20	19/45

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Table II. Polymorphisms noted in the full length cDNA sequences [Poly (A)$^+$ RNA was isolated [D.H. MacLennan and S. de Leon, Methods Enzymol. **96**:570 (1983)] from skeletal muscle from a proven N/N Yorkshire pig and from a proven

5 n/n Pietrain pig. Complementary cDNA was synthesized using the cDNA Synthesizing System supplied by Bethesda Research Laboratories (BRL), and cDNA libraries were constructed in λ gt10 (BRL) and λ ZAP (Stratagene) according to the manufacturer. Priming of n/n Pietrain

10 poly(A)$^+$ RNA (10 ug) for cDNA synthesis was carried out with 100 ng oligo d(T)$_{17}$, a 24 nucleotide primer complementary to the human skeletal muscle ryanodine reCeptor nucleotides 1243 to 1266 (10) and 18 nucleotide primers complementary to rabbit skeletal muscle ryanodine

15 receptor nucleotides 3499 to 3516, 4900 to 4917 and 9123 to 9140 (9). Priming of N/N Yorkshire poly(A)$^+$ RNA (10 ug) was carried out with the same set of oligonucleotides, plus 24 nucleotide sequences complementary to human nucleotides 4000 to 4023, 5859 to

20 5882 and 11099 to 11122. Restriction endonuclease fragments from the human skeletal muscle ryanodine receptor cDNA were used as probes to screen porcine cDNA libraries with filter washing at 60° [T. Maniatis, E.F. Fritsch, and J. Sambrook, Molecular Cloninc: A

25 Laboratory Manual (Cold Spring Harbor Laboratory, New York (1982)]. The polymerase chain reaction [R.K. Saiki et al., Science **239**, 487 (1988)] was used to isolate nucleotides 12502 to 12567 in Pietrain cDNA and nucleotides -24 to 448 in Yorkshire cDNA. All cDNAs were

30 subcloned into the Bluescript vector (Stratagene) for sequence analysis [F. Sanqer, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U.S.A. **74**:5463 (1977)] with the T7 sequencing kit (Pharmacia). Five independent PCR products were sequenced for detection of potential

35 artifactual sequences] that encode ryanodine receptors from N/N Yorkshire and n/n Pietrain swine. The left column identifies nucleotide numbers in which

polymorphisms were found by sequencing (with the exception of nucleotides 1843, 4332 and 13878, these were not confirmed by alternate tests) and amino acids numbers potentially altered. Alteration of nucleotide 1843

5   deletes a Hin P1 site (GCGC to GTGC); of 4332 creates a Ban II site (GAACCC to GAGCCC); and of 13878 deletes an Rsa I site (GTAC to ATAC) in the MH haplotype. The center (Yorkshire) and right (Pietrain) columns identify the nucleotide polymorphisms (bold) within their

10   respective codons and the effect of the polymorphism on the amino acid encoded. Nucleotides encoding MHN and MHS ryanodine receptors are numbered positively in the 5′ to 3′ orientation, beginning with residue 1 of the ATG initiator methionine. Amino acids are numbered

15   positively beginning with the initiator methionine.

Table III. Haplotype and genotype analysis of the RYRI gene in six swine breeds. PCR amplified products that surround the mutation at nucleotide 1843 (Hin PI) or

20   polymorphic sites at nucleotides 4332 (Ban II) and 13878 (Rsa I) were analysed for the presence (+) or absence (-) of appropriate restriction endonuclease sites (21). On the basis of the results of Hin P1 digestion, animals were grouped as T/T (proven n/n); C/T (probable N/n); and

25   C/C (probable N/N). The Hin PI⁻ Ban II⁺ Rsa I⁻ haplotype was identical for the two n/n chromosomes in each individual in each breed, and, therefore, chromosomes were counted individually in the n/n row. The haplotypes of the probable N/n and N/N chromosomes differed among

30   individuals and breeds and were counted as chromosome pairs.

We therefore have a reasonable prediction that the same defective gene will be found in any other breed of pig, especially where MH has been associated with lean

35   body mass, probably by the introduction into the breeding stock of individuals from other breeds carrying the original MH gene. The availability of probes 17-C and

15-T should make it feasible to eliminate the defective RYR1 gene from each of these breeds within a short period, if that is desired by pig breeders.

One major application of the DNA sequence information of the normal and mutant MH genes is in the area of genetic testing, carrier detection and herd diagnosis. Pigs (male and female) carrying mutations in the ryanodine receptor may be detected at the DNA level with the use of a variety of techniques. The genomic DNA used for the diagnosis may be obtained from body cells, such as those present in peripheral blood, urine, saliva, tissue biopsy and surgical specimen. The DNA may be used directly for detection of specific sequence or may be amplified enzymatically *in vitro* by using PCR [Saiki et al. *Science* 230:1350-1353, (1985), Saiki et al. *Nature* 324:163-166 (1986)] prior to analysis. RNA or its cDNA form may also be used for the same purpose. Recent reviews of this subject have been presented by Caskey, [*Science* 236:1223-8 (1989)] and by Landegren et al [*Science* 242:229-237 (1989).

The detection of specific DNA sequence may be achieved by methods such as hybridization using specific oligonucleotides [Wallace et al. *Cold Spring Harbour Symp. Quant. Biol.* 51:257-261 (1986)], direct DNA sequencing [Church and Gilbert, *Proc. Nat. Acad. Sci. USA* 81:1991-1995 (1988)], the use of restriction enzymes [Flavell et al. *Cell* 15:25 (1978), Geever et al *Proc. Nat. Acad. Sci. USA* 78:5081 (1981)], discrimination on the basis of electrophoretic mobility in gels with denaturing reagent [Myers and Maniatis, *Cold Spring Harbour Sym. Quant. Biol.* 51:275-284 (1986)], temperature gradient gel electrophoresis, RNase protection [Myers, R. M., Larin, J., and T. Maniatis *Science* 230:1242 (1985)], chemical cleavage [Cotton et al *Proc. Nat. Acad. Sci. USA* 85:4397-4401, (1985)] and the ligase-mediated detection procedure [Landegren et al *Science* 241:1077 (1988)].

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labelled radioactively with isotopes (such as ^{32}P) or non-radioactively (with tags such as

5   biotin [Ward and Langer et al. *Proc. Nat. Acad. Sci. USA* 78:6633-6657 (1981)], and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these specific sequences are

10 visualized by methods such as autoradiography or fluorometric (Landegren et al, 1989, *supra*) or colorimetric reactions [Gebeyehu et al *Nucleic Acids Research* 15:4513-4534 (1987)]. As is appreciated by those skilled in the art, the sequence length of the

15 oligonucleotides is sufficient to ensure hybridization of the probe. Usually the oligos of subject material are approximately 12 to 20 bp and up; for example, the specific 15-C and 17-T probes of the preferred embodiment of the invention.

20   Sequence differences between normal and mutants may be revealed by the direct DNA sequencing method of Church and Gilbert (*supra*). Cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR

25 [Wrichnik et al, *Nucleic Acids Res.* 15:529-542 (1987); Wong et al, *Nature* 330:384-386 (1987); Stoflet et al, *Science* 239:491-494 (1988)]. In the latter procedure, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or

30 single-stranded template generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent-tags.

        Sequence alterations may occasionally generate

35 fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by conventional gel-blot hybridization

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[Southern, *J. Mol. Biol.* 98: 503 (1975)]. DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of corresponding restriction fragment numbers. Genomic DNA samples may

5   also be amplified by PCR prior to treatment with the appropriate restriction enzyme; fragments of different sizes are then visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

     The discovery of a unique mutation in the pig

10   ryanodine receptor gene has uncovered that the mutation deletes a *HinPI* restriction enzyme site and creates a *HgiAI* site at nucleotide position 1843, (hereinafter referred to as site 1). *HinPI* digestion of the mutant sequence would not result in cleavage at nucleotide

15   position 1843 while digestion with *HgiAI would*. This leads to a restriction enzyme recognition site for DNA analysis. As can be appreciated, this region of the gene may be amplified by PCR techniques. The cDNA, as shown in Figure 2, consists of several exons, with the intron

20   information deleted. As is appreciated by those skilled in the art, intron information to either or both sides of the exon containing position 1843 may be used in the PCR amplification of this portion of the genomic DNA. It is also possible to select a portion of the intron which

25   would carry a cleavable *HinPI* or *HgiAI* site (hereinafter referred to as site 2). Site 2 would act as a built-in control to monitor whether the conditions for each restriction endonucleous digestion were appropriate. Thus for *HinPI*, 100% digestion of site 2 combined with

30   100% digestion of site 1 would indicate homozygous normal; 100% digestion of site 2 combined with 0% digestion of site 1 would indicate homozygous MH; 100% digestion of site 2 combined with 50% digestion of site 1 would indicate a heterozygo. Less than 100% of site 2

35   would require a repeat digestion. For *HgiAI*, 100% digestion at sites 2 and 1 would indicate homozygous MH; 100% digestion at site 2 and 0% digestion at site 1 would

indicate homozygous normal; 100% digestion at site 2 and
50% digestion at site 1 would indicate a heterozygate;
partial digestion would require a repeat digestion.

5   We tested for the presence of the mutation either by
oligonucleotide hybridization or by analysis of
restriction endonuclease digestion patterns because the
mutation deletes a *HinPl* site and, simultaneously,
creates a *HgiAI* site in the porcine gene. The use of
restriction endonuclease digestion as the basis for
10   genotype analysis is superior to oligonucleotide
hybridization because it is simpler, cheaper and less
hazardous, and because it has the potential for greater
accuracy, particularly in cases where samples are
partially contaminated. We recognized the desirability,
15   however, of improving our restriction endonuclease based
test for the mutation in two ways. First, it was
important to obtain a larger DNA fragment that would
absorb more stain so that fragments and subfragments
would be more readily visualized following gel
20   electrophoresis. Second, since the test must
differentiate digestion of one allele for heterozygotes
from partial digestion of normal or MH genotypes, a
"built in control" digestion site which should always be
100% digested would be an important feature of a simple
25   but reliable test.

Our strategy in achieving these goals was to isolate
and sequence intron DNA lying between the exon containing
the mutation site and its two flanking exons. We then
designed PCR primers that would allow amplification of
30   fragments between 500 and 1000 bp which would contain
either a constant *HinPI* or *HgiAI* site in the porcine *RYR1*
gene which would be sufficiently distant from the
polymorphic mutation site that cleavage at one or both
sites could be readily detected following gel
35   electrophoresis. The pig intron sequences flanking the
exon containing the mutation site, were PCR amplified
with appropriate primers within the predicted flanking

40

porcine exons. Genomic DNA was isolated from the blood
of a Yorkshire pig. The forward primer [5'
-ATCTCTAGAGACAAGCATGGGAGGAACCACAAG-3'] corresponded to
nucleotides 1771 to 1794, while the reverse primer [5'

5   -ATCTCTAGACCCACAAAGATGTTGGGGCGGATG-3'] corresponded to
nucleotides 1929 to 1952 in pig ryanodine receptor cDNA
[Fujii et al., "Identification of a Mutation in the
Porcine Ryanodine Receptor Associates with Malignant
Hyperthermia. *Science*, 253:448-451, 1991]. The first

10   nine nucleotides constituted an *XbaI* restriction
endonuclease site which facilitates subcloning.
Amplification was carried out in the PCR buffer provided
by Perkin Elmer Cetus, containing 1 mM $MgCl_2$ and 100 ng
each of primers, for 35 cycles using a step program of

15   denaturation at 94°C for 1 min, annealing at 55°C for 2
min and extension at 72°C for 3 min in a DNA Thermal
Cycler (Perkin-Elmer Cetus). The 1.6 kbp PCR product
which we obtained was digested with appropriate
restriction endonucleases and subcloned into the

20   Bluescript Vector. Sequencing of the subclones was
performed on both strands as described above. The pig
genomic sequence lying between the exon containing the
mutation and its flanking exons is shown in Figure 6.
        The exon which contains the mutation site was 134 bp

25   long. The nucleotide sequences observed at 5' donor and
3' acceptor splice sites in each of the four introns were
in agreement with the consensus sequences reported
previously (Breathnach and Chambon, "Organization and
Expression of Eucaryotic Split Genes Coding for

30   Proteins", *Annu. Rev. Biochem.*, 50:349-383, 1981). The
mutation of the sequence GCGCTC to GTGCTC deletes a *HinPI*
site and creates a *HgiAI* restriction endonuclease site in
pig (Fujii et al., supra; Otsu et al., "Cosegregation of
Porcine Malignant Hyperthermia and a Probable Causal

35   Mutation in the Skeletal Muscle Ryonodine Receptor Gene
in Backcross Families, *Genomics* 11 in press, 1991). In
pig, a constant *HgiAI* site was located 358 bp upstream of

the polymorphic mutation site. Accordingly, primers to be used in the MH detection assay were chosen so that tne PCR products would include these sites as internal controls for restriction endonuclease digestion.

5　Diagrammatic representation of the amplified pig PCR products is presented in Figure 7.

A pig sequence of 659 bp between nucleotides overlined in Figure 6 was amplified for analysis by *HgiAI* digestion from genomic DNA from homozygous MH,

10　heterozygous MH and homozygous normal pigs (Fujii et al. supra). The amplification buffer contained 1 mM $MgCl_2$, 200-500 ng of pig genomic DNA and 100 ng each of the forward (5' -TCCAGTTTGCCACAGGTCCTACCA-3') and reverse primers (5'-TTCACCGGAGTGGAGTCTCTGAGT-3'). Amplification

15　conditions were those defined above, except that the annealing temperature was 53°C.

In Figure 8 we illustrate the *HgiAI* restriction endonuclease digestion patterns derived from the 659 bp products amplified from normal (N/N), homozygous MH (n/n)

20　and heterozygous (N/n) porcine genotypes, from DNA from a n/n individual BC69 (Otsu et al., supra) contaminated either manually or naturally (possible chimaerism; McFee et al., "An Intersex Pig with XX/YY Leucocyte Mosaicism, *Can. J. Genet Cytol.*, **8**:502-505, 1966) with N/n DNA, and

25　from n/n DNA partially digested by *HgiAI*. In each case, the first lane (-) represents the PCR amplified product and the second lane (+) represents the same product after digestion with *HgiAI*. DNA from the N/N individual gave bands of 524 and 135 bp when digested with *HgiAI*, while

30　DNA from the n/n individual gave bands of 358, 166 and 135 bp, with the 166 bp band of slightly greater intensity than the 135 bp band. Digestion of DNA from the heterozygous individual gave bands of 524, 358, 166 and 135 bp. In this case, the intensity of the 358 bp

35　band was less than that of the 524 bp band and the intensity of the 166 bp band was less than that of the 135 bp band. In sample BC69, contamination of n/n DNA

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with a lesser amount of N/n DNA was detectable after digestion because the 358 bp band was of about equal intensity with the 524 bp band and the 166 bp band was of only slightly lower intensity than the 135 bp band. Thus

5 it is possible to detect contamination of samples by comparison of band intensities. With sample BC152, we illustrate a case where remnants of the 659 bp band containing the constant *HgiAI* site indicate incomplete digestion of a n/n DNA, requiring a repeat analysis.

10 While these modifications in PCR amplification and restriction endonuclease digestion make the diagnostic test for porcine MH more accurate and reliable, its adaptation to large scale testing requirements may be enhanced by the following. We have evaluated the rapid

15 isolation of genomic DNA from porcine blood by the method of Kawasaki (1990 "Sample Preparation from Blood, Cells, and other Fluids" In: "PCR Protocols" (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J.) pp 142-152, Academic Press, New York). Fifty μl of EDTA-treated

20 blood were added to 0.5 ml of a solution of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, (TE) in a 1.5 ml Eppendorf centrifuge tube and spun for 10 sec. The pellet was washed 3 times with 0.5 ml of TE by vortexing and spinning and resuspended in 100 μl of gelatin-free Perkin

25 Elmer Cetus PCR buffer containing 0.5% Tween 20 and 100 μg/ml of Proteinase K. After incubation at 56°C for 30 min to complete digestion and 10 min at 95° to inactivate the protease, 10 μl of this solution was used for PCR amplification, as outlined above. The product was

30 indistinguishable from the product obtained from DNA isolated conventionally (Miller et al., "A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells", *Nucleic Acids Res.*, 16:1215, 1988).

We have also evaluated the potential of agarose

35 rather than acrylamide gels for separation of the various fragments, since the use of horizontal agarose gels is more readily adapted to large scale analysis than the use

of thin vertical acrylamide gels. We have found that the
use of a 3% Nu Sieve 3:1 Agarose gel (FMC Bioproducts) in
TBE buffer, pH 8.5, (Maniatis et al., "Molecular Cloning,
a Laboratory Manual", Cold Spring Harbor Laboratory

5　Press, Cold Spring Harbor, NY, 1982) provides adequate
separation of the bands between 940 and 135 bp that are
generated in these tests. These bands can be detected by
staining with ethidium bromide added to the sample
buffer.

10　　These additional refinements provides a rapid,
accurate, non-invasive, inexpensive, DNA based test for
detection of a probable causative mutation for MH in both
porcine and human genomic DNA. The test can be used for
reliable, large scale screening for MH in populations of

15　both species.

　　Genetic testing based on DNA sequence differences
may be achieved by detection of alteration in
electrophoretic mobility of DNA fragments in gels with or
without denaturing reagent. Small sequence deletions and

20　insertions can be visualized by high resolution gel
electrophoresis. For example, the PCR product with the 1
bp substitution is clearly distinguishable from the
normal sequence on an 8% non-denaturing polyacrylamide
gel. DNA fragments of different sequence compositions

25　may be distinguished on denaturing formamide gradient gel
in which the mobilities of different DNA fragments are
retarded in the gel at different positions according to
their specific "partial-melting" temperatures [Myers,
supra). In addition, sequence alterations, may be

30　detected as changes in the migration pattern of DNA
heteroduplexes in non-denaturing gel electrophoresis, as
have been detected for the 1 bp mutation and in other
experimental systems [Nagamine et al, *Am. J. Hum. Genet,*
45:337-339 (1989)]. Alternatively, a method of detecting

35　a mutation comprising a single base substitution or other
small change could be based on differential primer length
in a PCR. For example, one invariant primer could be

used in addition to a primer specific for a mutation.
The PCR products of the normal and mutant genes can then
be differentially detected in acrylamide gels.

Sequence changes at specific locations may also be
5   revealed by nuclease protection assays, such as RNase
(Myers, *supra*) and S1 protection Berk, A. J., and P. A.
Sharpe, *Proc. Nat. Acad. Sci. USA, 75*:1274 (1978)], the
chemical cleavage method (Cotton, *supra*) or the ligase-
mediated detection procedure (Landegren *supra*).

10   In addition to conventional gel-electrophoresis and
blot-hybridization methods, DNA fragments may also be
visualized by methods where the individual DNA samples
are not immobilized on membranes. The probe and target
sequences may be both in solution or the probe sequence
15   may be immobilized [Saiki et al, *Proc. Natl. Acad. Sci
USA, 86*:6230-6234 (1989)]. A variety of detection
methods, such as autoradiography involving radioisotopes,
direct detection of radioactive decay (in the presence or
absence of scintillant), spectrophotometry involving
20   colorigenic reactions and fluorometry involving
fluorogenic reactions, may be used to identify specific
individual genotypes.

In summary, the screening method comprises the steps
of:
25   providing a biological sample of the subject to be
screened; and providing an assay for detecting in the
biological sample, the presence of at least a member from
the group consisting of the normal porcine ryanodine
receptor (RYR1), normal RYR1 products, an MH mutant RYR1,
30   MH mutant RYR1 products and mixtures thereof.

The method may be further characterized by including
at least one more nucleotide probe which is a different
DNA sequence fragment of, for example, the DNA of Figure
2, or a different DNA sequence fragment of pig chromosome
35   6 and located to either side of the DNA sequence of
Figure 2.

A kit, according to an embodiment of the invention, suitable for use in the screening technique and for assaying for the presence of the RYR1 gene by an immunoassay comprises:

5   (a)   an antibody which specifically binds to a gene product of the RYR1 gene;

(b)   reagent means for detecting the binding of the antibody to the gene product; and

(c)   the antibody and reagent means each being
10   present in amounts effective to perform the immunoassay.

The kit for assaying for the presence for the RYR1 gene may also be provided by hybridization techniques. The kit comprises:

(a)   oligonucleotide sequences for PCR priming of
15   the appropirate porcine genomic sequence;

(b)   oligonucleotide probes which specifically bind to the RYR1 gene;

(c)   reagent means for detecting the hybridization of the oligonucleotide probes to the RYR1 gene; and
20   (d)   the probes and reagent means each being present in amounts effective to perform the hybridization assay.

A significant use for this invention is determining whether elimination of the defective gene might also decrease lean body mass in the various pig breeds. It is
25   possible that there is an association between a defective RYR1 gene and lean body mass, since the hypersensitive excitation-contraction coupling, which is characteristic of MHS animals [O'Brien (1987) *supra*; O'Brien (1986) *supra* and O'Brien (1990) *supra*], is analogous to chronic
30   function overload [O'Brien et al (1991) *Can. J. Physol Pharmacol* 69] which results in muscle hypertrophy. Furthermore, this may divert energy to Ca^{2+} pumping and muscle contraction and away from fat storage. If this were the case, then it would be advantageous to
35   reintroduce the defective RYR1 gene selectively into breeding stock. For example, crosses of MH/MH X N/N would yield F1 generation market animals which might all

●   ●

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have superior lean body mass and acceptable meat quality
[Elizondo et al (1977) *J. Anim. Sci.* 45:1272-1279]. The
availability of the probes, according to this invention,
for accurate diagnosis of breeding stocks now make such a
5   breeding program feasible.

It is also feasible to develop polyclonal or
monoclonal antibodies against the specific peptide
sequences surrounding the mutation leading to MH so that
these differential antibodies can be used to detect
10   differences in normal and mutant ryanodine receptor
proteins. It is likely that all MHS swine will have the
same or another section of the protein sequence either
altered or missing. It is possible to express specific
DNA sequences in biological systems [e.g. Clarke et al
15   (1990) *J. Biol. Chem.*265:17405-17408] to make either
unique peptide sequences or to express the sequences from
a fused gene which will make a fusion peptide. It is
also possible to synthesize peptides chemically and to
link them to other proteins. Any of these peptide forms
20   can be potential antigens for the production of specific
antibodies. Thus antibodies raised to the altered
sequence (mutant MH) will show positive immunostaining on
muscle sections in MH pigs and heterozygous pigs, since
they will react with the antigen in the muscle. No
25   staining should be observed in normal porcine individuals
but antibodies raised against the normal sequence will
stain normal muscle. Heterozygous pigs should be
distinguished from homozygous affected pigs because they
will always react with the antibody raised against the
30   normal peptide. Similarly, these reactions could be
observed with the more sensitive technique of
immunoblotting [Towbin et al. (1979) *Proc. Natl. Acad.
Sci. USA* 76:4350-4354] of extracts of muscle protein.

As mentioned, antibodies to epitopes of the porcine
35   RYR1 protein are raised to provide extensive information
on the characteristics of the protein and other valuable
information which includes:

1.   To enable visualization of the protein in cells
and tissues in which it is expressed by
immunoblotting ("Western blots") following
polyacrylamide gel electrophoresis. This allows an
estimation of the molecular size of the mature
protein including the contribution from the cells of
post-translationally added moieties including
oligosaccharide chains and phosphate groups, for
example. Immunocytochemical techniques including
immunofluorescence and immuno-electronmicroscopy can
be used to establish the subcellular localization of
the protein in cell membranes. The antibodies can
also be used to provide another technique in
detecting any of the other RYR1 mutations in perhaps
othe pig breeds which result in the synthesis of a
protein with an altered size.

2.   Antibodies to distinct domains of the protein
can be used to determine the topological arrangement
of the protein in the cell membrane. This provides
information on segments of the protein which are
accessible to externally added modulating agents for
purposes of drug therapy.

3.   The structure-function relationships of
portions of the protein can be examined using
specific antibodies. For example, it is possible to
introduce into cells antibodies recognizing each of
the charged cytoplasmic loops which join the
transmembrane sequences as well as portions of the
nucleotide binding folds and the R-domain. The
influence of these antibodies on functional
parameters of the protein provide insight into cell
regulatory mechanisms and potentially suggest means
of modulating the activity of the defective protein
in an MH pig.

4.   Antibodies with the appropriate avidity also
enable immunoprecipitation and immuno-affinity
purification of the protein. Immunoprecipitation

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will facilitate characterization of synthesis and
post translational modification including ATP
binding and phosphorylation. Purification will be
required for studies of protein structure and for
5   reconstitution of its function, as well as protein
based therapy.

It is possible to raise polyclonal antibodies
specific for both fusion proteins containing portions of
the RYR1 protein and peptides corresponding to short
10   segments of its sequence. Monoclonal antibodies can be
similarly raised to other domains of the RYR1 protein.
It is preferred, as is appreciated by those skilled in
the art, that the protein fragment should contain
approximately 75 to 100 amino acids of the subject
15   material.

As with the generation of polyclonal antibodies,
immunogens for the raising of monoclonal antibodies
(mAbs) to the RYR1 protein may be via bacterial fusion
proteins [Smith et al, *Gene* 67:31 (1988)] containing
20   portions of the RYR1 polypeptide or synthetic peptides
corresponding to short (12 to 25 amino acids in length)
segments of the sequence. The essential methodology is
that of Kohler and Milstein [*Nature* 256:495 (1975)].

Balb/c mice are immunized by intraperitoneal
25   injection with 500 μg of pure fusion protein or synthetic
peptide in incomplete Freund's adjuvant. A second
injection is given after 14 days, a third after 21 days
and a fourth after 28 days. Individual animals so
immunized are sacrificed one, two and four weeks
30   following the final injection. Spleens are removed,
their cells dissociated, collected and fused with Sp2/O-
Ag14 myeloma cells according to Gefter et al, *Somatic
Cell Genetics* 3:231 (1977). The fusion mixture is
distributed in culture medium selective for the
35   propagation of fused cells which are grown until they are
about 25% confluent. At this time, culture supernatants
are tested for the presence of antibodies reacting with a

particular RYR1 antigen. An alkaline phosphatase
labelled anti-mouse second antibody is then used for
detection of positives. Cells from positive culture
wells are then expanded in culture, their supernatants

5   collected for further testing and the cells stored deep
frozen in cryoprotectant-containing medium. To obtain
large qu:ntities of a mAb, producer cells are injected
into the peritoneum at 5×10^6 cells per animal, and
ascites fluid is obtained. Purification is by

10   chromatography on Protein G- or Protein A-agarose
according to Ey et al, *Immunochemistry* 15:429 (1977).

Reactivity of these mAbs with the RYR1 protein is
confirmed by polyacrylamide gel electrophoresis of
membranes isolated from epithelial cells in which it is

15   expressed and immunoblotted [Towbin et al, *Proc. Natl.
Acad. Sci. USA* 76:4350 (1979)].

In addition to the use of monoclonal antibodies
specific for each of the different domains of the RYR1
protein to probe their individual functions, other mAbs,

20   which can distinguish between the normal and mutant forms
of RYR1 protein, are used to detect the mutant protein in
muscle cells.

Antibodies capable of this distinction are obtained
by differentially screening hybridomas from paired sets

25   of mice immunized with a peptide fragment containing the
arginine at amino acid position 615 or cysteine at amino
acid position 615.

Antibodies to normal and MH versions of RYR1 protein
and of segments thereof are used in diagnostically

30   immunocytochemical and immunofluorescence light
microscopy and immunoelectron microscopy to demonstrate
the tissue, cellular and subcellular distribution of RYR1
within the muscle of MH pigs, carriers and non-MH
individuals.

35   Other modes of expression in heterologous cell
system also facilitate dissection of structure-function
relationships. The complete RYR1 DNA sequence ligated

into a plasmid expression vector is used to transfect
cells so that its influence on ion fluxes in the cell can
be assessed.   Plasmid expression vectors containing part
of the normal RYR1 sequence along with portions of

5   modified sequence at selected sites can be used *in vitro*
mutagenesis experiments performed in order to identify
those portions of the RYR1 protein which are crucial for
regulatory function.

        The DNA sequence can be manipulated in studies to

10   understand the expression of the gene and its product,
and, to achieve production of large quantities of the
protein for functional analysis, antibody production, and
pig therapy.   The changes in the sequence may or may not
alter the expression pattern in terms of relative

15   quantities, tissue-specificity and functional properties.
The partial or full-length cDNA sequences, which encode
for the subject protein, unmodified or modified, may be
ligated to bacterial expression vectors such as the pRIT
[Nilsson et al. *EMBO J.* 4:1075-1080 (1985)], pGEX [Smith

20   and Johnson, *Gene* 67: 31-40 (1988)] or pATH [Spindler et
al. *J. Virol.* 49: 32-141 (1984)] plasmids which can be
introduced into *E. coli* cells for production of the
corresponding proteins which may be isolated in
accordance with the standard protein purification

25   procedures.   The DNA sequence can also be transferred
from its existing context to other cloning vehicles, such
as other plasmids, bacteriophages, cosmids, animal virus,
yeast artificial chromosomes (YAC) [Burke et al. *Science*
236:806-812, (1987)], somatic cells, and other simple or

30   complex organisms, such as bacteria, fungi [Timberlake
and Marshall, *Science* 244:1313-1317 (1989)],
invertebrates, plants [Gasser and Fraley, *Science* 244:
1293 (1989)], and pigs [Pursel et al. *Science* 244:1281-
1288 (1989)].

35   For expression in mammalian cells, the cDNA sequence
may be ligated to heterologous promoters, such as the
simian virus (SV) 40, promoter in the pSV2 vector

[Mulligan and Berg, *Proc. Natl. Acad. Sci USA*, <u>78</u>:2072-
2076 (1981)] and introduced into cells, such as monkey
COS-1 cells [Gluzman, *Cell*, <u>23</u>:175-182 (1981)], to
achieve transient or long-term expression. The stable
5   integration of the chimeric gene construct may be
maintained in mammalian c.lls by biochemical selection,
such as neomycin [Southern and Berg, *J. Mol. Appln.
Genet.* <u>1</u>:327-341 (1982)] and mycophoenolic acid [Mulligan
and Berg, *supra*].

10   DNA sequences can be manipulated with standard
procedures such as restriction enzyme digestion, fill-in
with DNA polymerase, deletion by exonuclease, extension
by terminal deoxynucleotide transferase, ligation of
synthetic or cloned DNA sequences, site-directed
15   sequence-alteration via single-stranded bacteriophage
intermediate or with the use of specific oligonucleotides
in combination with PCR.

The cDNA sequence (or portions derived from it), or
a mini gene (a cDNA with an intron and its own promoter)
20   is introduced into eukaryotic expression vectors by
conventional techniques. These vectors are designed to
permit the transcription of the cDNA in eukaryotic cells
by providing regulatory sequences that initiate and
enhance the transcription of the cDNA and ensure its
25   proper splicing and polyadenylation. Vectors containing
the promoter and enhancer regions of the simian virus
(SV)40 or long terminal repeat (LTR) of the Rous Sarcoma
virus and polyadenylation and splicing signal from SV 40
are readily available [Mulligan et al *Proc. Natl. Acad.
30   Sci. USA 78:1078-2076, (1981); Gorman et al Proc Natl.
Acad. Sci USA* 79: 6777-6781 (1982)]. The level of
expression of the cDNA can be manipulated with this type
of vector, either by using promoters that have different
activities (for example, the baculovirus pAC373 can
35   express cDNAs at high levels in *S. frungiperda* cells [M.
D. Summers and G. E. Smith in, Genetically Altered
Viruses and the Environment (B. Fields, et al, eds.) vol.

●   ●

22 no 319-328, Cold Spring Harbour Laboratory Press, Cold
Spring Harbour, New York, 1985] or by using vectors that
contain promoters amenable to modulation, for example the
glucocorticoid-responsive promoter from the mouse mammary
5   tumor virus [Lee et al, *Nature* 294:228 (1982)]. The
    xpression of the cDNA can be monitored in the recipient
    cells 24 to 72 hours after introduction (transient
    expression).

         In addition, some vectors contain selectable markers
10   [such as the *gpt* [Mulligan et Berg *supra*] or *neo*
    [Southern and Berg *J. Mol. Appln. Genet* 1:327-341 (1982)]
    bacterial genes that permit isolation of cells, by
    chemical selection, that have stable, long term
    expression of the vectors (and therefore the cDNA) in the
15   recipient cell. The vectors can be maintained in the
    cells as episomal, freely replicating entities by using
    regulatory elements of viruses such as papilloma [Sarver
    et al *Mol. Cell Biol.* 1:486 (1981)] or Epstein-Barr
    (Sugden et al *Mol. Cell Biol.* 5:410 (1985)].
20   Alternatively, one can also produce cell lines that have
    integrated the vector into genomic DNA. Both of these
    types of cell lines produce the gene product on a
    continuous basis. One can also produce cell lines that
    have amplified the number of copies of the vector (and
25   therefore of the cDNA as well) to create cell lines that
    can produce high levels of the gene product [Alt et al.
    J. Biol. Chem. 253:1357 (1978)].
         The transfer of DNA into eukaryotic, in particular
    human or other mammalian cells for purposes of disease
30   study is now a conventional technique. The vectors are
    introduced into the recipient cells as pure DNA
    (transfection) by, for example, precipitation with
    calcium phosphate [Graham and vander Eb, *Virology* 52:466
    (1973) or strontium phosphate [Brash et al *Mol. Cell
35   *Biol.* 7:2013 (1987)], electroporation [Neumann et al *EMBO
    J* 1:841 (1982)], lipofection [Felgner et al *Proc Natl.*
    Acad. Sci USA 84:7413 (1987)], DEAE dextran [McCuthan et

al *J. Natl Cancer Inst.* <u>41</u>:351 1968)], microinjection
[Mueller et al *Cell* <u>15</u>:579 1978)], protoplast fusion
[Schafner, *Proc Natl. Aca. Sci USA* <u>72</u>:2163] or pellet
guns [Klein et al, *Nature* <u>327</u>:70 (1987)]. Alternatively,
5 the cDNA can be introduced by infection with virus
vectors. Systems are developed that use, for example,
retroviruses [Bernstein et al. *Genetic Eng. neering* <u>7</u>:
235, (1985)], adenoviruses [Ahmad et al *J. Virol* <u>57</u>:267
(1986)] or Herpes virus [Spaete et al *Cell* <u>30</u>:295
10 (1982)].

These eukaryotic expression systems can be used for
many studies of the MH gene and the RYR1 product, not
only as associates with pigs, but also to provide insight
into human MHS. These include, for example: (1)
15 determination that the gene is properly expressed and
that all post-translational modifications necessary for
full biological activity have been properly completed;
(2) production of large amounts of the normal protein for
isolation and purification (3) to use cells expressing
20 the RYR1 protein as an assay system for antibodies
generated against the RYR1 protein or an assay system to
test the effectiveness of drugs, (4) study the function
of the normal complete protein, specific portions of the
protein, or of naturally occurring or artificially
25 produced mutant proteins. Naturally occurring mutant
proteins exist in pigs with MH while artificially
produced mutant protein can be designed by site directed
sequence alterations. These latter studies can probe the
function of any desired amino acid residue in the protein
30 by mutating the nucleotides coding for that amino acid.

The recombinant cloning vector, according to this
invention, then comprises the selected DNA of the DNA
sequences of this invention for expression in a suitable
host. The DNA is operatively linked in the vector to an
35 expression control sequence in the recombinant DNA
molecule so that normal RYR1 polypeptide can be
expressed. The expression control sequence may be

selected from the group consisting of sequences tnat
control the expression of genes of prokaryotic or
eukaryotic cells and their viruses and combinations
thereof. The expression control sequence may be
5 specifically selected from the group consisting of the
lac system, the *rp* system, the *tac* system, the *trc*
system, major opc.rator and promoter regions of phage
lambda, the control region of fd coat protein, the early
and late promoters of SV40, promoters derived from
10 polyoma, adenovirus, retrovirus, baculovirus and simian
virus, the promoter for 3-phosphoglycerate kinase, the
promoters of yeast acid phosphatase, the promoter of the
yeast alpha-mating factors and combinations thereof.

The host cell, which may be transfected with the
15 vector of this invention, may be selected from the group
consisting of *E. coli, Pseudomonas, Bacillus subtilis,*
Bacillus stearothermophilus or other bacilli; other
bacteria; yeast; fungi; insect; mouse or other animal; or
plant hosts; or human tissue cells.

20 It is appreciated that for the mutant DNA sequence
similar systems are employed to express and produce the
mutant product.

The DNA sequence disclosed herein can be manipulated
in order to achieve expression and production of larger
25 quantities of the protein for functional analysis and
antibody production. Partial or full length cDNA
sequences, which encode porcine RYR1 may be ligated to
bacterial expression vectors (for example, pRIT, pGEX, or
pATH). The DNA can also be transferred from its existing
30 context to other cloning vehicles, such as other
plasmids, bacteriophages, cosmids, animal virus, yeast
artificial chromosomes, somatic cells, and other simple
or complex organisms, such as bacteria, fungi,
invertebrates, or plants. The cDNA- sequence (or
35 portions thereof) or a mini gene can be introduced into
eukaryotic expression vectors by standard techniques.
The expression vectors can also be introduced into

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recipient cells by conventional techniques, including
transfection, calcium or strontium phosphate
precipitation, electroporation, microinjection,
protoplast fusion, or virus vectors.

5   Antibodies to various epitopes of the porcine RYR1
protein can be raised to provide extensive information on
the characteristics of the protein and other valuable
information which can be used in immunoassays. The
antibodies may be raised to fusion proteins containing

10   defined portions of the porcine RYR1 polypeptide or may
be raised to protein fragments which are prepared by
chemical synthesis. As already noted, fusion proteins
may be developed by expression of vectors in suitable
hosts. As to the chemical synthesis of the proteins or

15   peptides, this is well within the skill of those
knowledgeable in the art.

        With the purified fusion protein, which may be
achieved by affinity chromatography, the protein
fragments can be injected into rabbits. Sera from the

20   rabbits immunized with the protein fragments are screened
in accordance with standard techniques to raise
polyclonal antibodies.

        DNA sequences may also be subjected to site directed
mutagenesis for comparative functional expression studies

25   [eg. Maruyama and MacLennan (1988) *Proc. Natl. Acad. Sci.
USA* 85:3314-3318). If the nature of the human and pig
mutations are different, it may be desirable to create
animal models which more closely resemble each human and
pig mutation. This could be achieved by site directed

30   mutagenesis in conjunction with homologous recombination
in embryonic stem cells, or by use of retrovirus vectors
to create transgenic animals.

        Our discovery of an altered nucleotide sequence
allows us to conduct a one-step, allele-specific PCR-

35   based diagnosis [Ballabio et al *Nature* 343:220 (1990)] of
MH from pig genomic DNA. Application of the one-step,
allele-specific PCR techniques to detect mutations has

already been exemplified in Ballabio et al (supra). In that particular application, forward primers were selected to detect the ΔF508 mutation in the cystic fibrosis gene. Each allele-specific primer had at its 3'
5   end a sequence corresponding to the normal allele or to the ΔF508 allele. Thus in such a test, nucleotides at the 3' end of forward or reverse primers impart allele specificity to priming. Similarly, with selected primers for differential detection of the MH allele, either
10   forward or reverse, the MH normal and mutated sequences are included towards the 3' end, as with the Ballabio et al (supra) technique.

As a second example, probes 17-C and 15-T, or modification of these probes to increase their
15   temperature sensitivity of hybridization, could be used as forward PCR primers with appropriate reverse PCR primers selected from Figure 2. Probe 17-C hybridizes to genomic DNA thereby permitting PCR amplification of the MHN sequence, but not the MHS sequence at temperatures
20   near 61°C. Conversely, probe 15-T hybridizes to genomic DNA thereby permitting PCR amplification of the MHS sequence, but not the MHN sequence, at temperatures near 55°C. Successful application of these primers, in separate reactions with the same genomic DNA, would yield
25   one product for 17-C with MHN DNA and no product for 15-T; no product for 17-C and a product for 15-T with MHS DNA; and products for both 17-C and 15-T with heterozygous DNA. The diagnosis would be complete following gel electrophoresis and analysis of the
30   presence or absence of a PCR product. Appropriate controls may be used in this diagnosis.

It is therefore appreciated that, in accordance with standard PCR techniques, a rapid, economical assay is provided to detect the presence of the T1843 mutation.
35   As is appreciated, the remaining forward or reverse primer may be located elsewhere along the RYR1 gene within standard PCR amplification distances. The

invention therefore contemplates any form of PCR
amplification technique where the forward or reverse
primer sequence includes either the normal C1843 base or
the mutant T1843 and the normal surrounding RYR1 sequence
5   as exemplified with primers 15-T and 17-C.

   The 17 polymorphisms that we have discovered are
potentially useful in further analysis of MH or other
genetic diseases involving the ryanodine receptors (for
example central core disease in humans has been linked to
10 the RYR1 gene: J. Mulley, personal communication). Such
polymorphisms can frequently be demonstrated to give rise
to restriction fragment length polymorphisms (RFLPs)
which have been useful in analysis of linkage between two
genes, one or both of which may give rise to a disease.
15 For example, if another, unknown mutation in the RYR1
gene should be found to give rise to MH, linkage studies
using probes for the presence of one or more of the
polymorphisms that we have described could provide a
useful means for following the inheritance of the
20 disease, indirectly, by the association of the
inheritance of a particular benign polymorphism
(haplotype) with inheritance of MH (or another disease).

   The following Examples are provided to demonstrate
preferred techniques for isolating the mutant RYR1 gene
25 and use of such genetic information.

Example 1

   Skeletal muscle from a homozygous MHN Yorkshire pig
and from a homozygous MHS Pietrain pig was dissected
immediately after sacrifice of the animal and frozen in
30 liquid nitrogen. Poly (A)$^+$ RNA was isolated as described
earlier [MacLennan et al, *Methods Enzymol.* 96:570-575
(1983)] and cDNA was synthesized using a kit supplied by
the Bethesda Research Laboratories (BRL) and cDNA
libraries were constructed in λgt10 (BRL) and λZAP
35 (Stratagene Cloning Systems) using protocols described in
their respective kits. Priming of 10 ug of MHS Pietrain
strain poly(A)$^+$ RNA for cDNA synthesis was carried out

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with 100 ng of each of oligo d(T)₁₇, a 24-mer
oligonucleotide complementary to human skeletal muscle
ryanodine receptor nucleotides 1243-1266 [Zarzato et al
supra] and 18-mer oligonucleotides complementary to

5 rabbit skeletal muscle ryanodine receptor nucleotides
9123-9140; 4900-4917 and 3499-3516 [Takeshima et al
(1990) *supra*]. Priming of 10 ug of the normal Yorkshire
strain poly(A)⁺ RNA was carried out with the same set of
oligonucleotide, plus 24-mer sequences complementary to

10 human nucleotides 4000-4023, 5859-5882 and 11099-11122.
Restriction endonuclease fragments from the human
skeletal muscle ryanodine receptor cDNA were used as
probes to screen porcine cDNA libraries as described
earlier [Maniatis et al (1982) *supra*, Otsu et al (1990)

15 *supra*], except that filter washing was at 60°. The
polymerase chain reaction was used to isolate nucleotides
12502 to 12567 in MHS Pietrain breed cDNA and nucleotides
-24 to 448 in MHN Yorkshire breed cDNA. Basic steps in
the reaction were as described previously [Elizondo et al

20 (1977) *supra*] except that the first strand was
synthesized from 1 ug of poly(A)⁺ RNA from either
Pietrain or Yorkshire breed muscle using 5 ng of random
primers (Pharmacia). The PCR reaction was carried out
with 1/500 of the initial cDNA, using 24-mer

25 oligonucleotide primers based on nucleotides 12400-12423
and 12613-12636 (Figure 2) for the Pietrain breed
amplification and nucleotides -24 to -1 and 597-620 for
the Yorkshire breed amplification. The reaction was
carried out for 35 cycles in a DNA Thermal Cycler⁹

30 (Perkin-Elmer Cetus) in 100 ul of the Gene Amp⁹ kit
(Perkin-Elmer Cetus) using a step program of annealing at
52° for 2 min; extension at 72° for 3 min and
denaturation at 95° for 40 sec with a final cycle of 7
min for extension. cDNA was subcloned into the

35 Bluescript Vector⁹ (Stratagene) for single strand,
dideoxy sequence [Sanger et al *Proc. Natl. Acad. Sci. USA*
<u>74</u>:5463-5467 (1977)] analysis using the T7 sequencing kit

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(Pharmacia). Five independent PCR products were
sequenced for detection of potential artifactual
sequences.

Example 2

5   Genomic DNA was isolated from fresh pig blood or
frozen pig muscle [Miller et al *Nucleic Acids Res.*16:1215
(1988)]. A specific region, of 74 bp lying between
residues 1811 and 1884 (see overlined sequences in Figure
2 which identify the PCR primers used in this reaction)
10   was PCR amplified from 2-5 ug of genomic DNA using 100 ng
of each primer. The forward primer used in PCR
amplification was synthesized by Kronem (Mississauga) and
was of the composition 5'-GTTCCCTGTGTGTGTGCAATGGTG-3',
corresponding to nucleotides 1811 to 1834 in Figure 2.
15   The reverse primer was of the composition 5'-
ATCTCTAGAGCCAGGGAGCAAGTTCTCAGTAAT-3'. The last 24
nucleotides in the reverse primer were complementary to
nucleotides 1861 to 1884 in Figure 2, while the first
nine nucleotides constitute an Xba restriction
20   endonuclease which was added to the primer for ease of
subcloning, but was not a prerequisite for this study.
These nine nucleotides can conveniently be left out of
future studies. The primers were annealed at 52° for 2
min, extended at 72° for 3 min and denatured at 95° for
25   40 sec with a final cycle of 7 min for extension in a DNA
thermal cycler (Perkin-Elmer Cetus). Part of the PCR
product was separated by electrophoresis to assure that
synthesis had occurred. An aliquot of 20 ul was
incubated for 30 min at 65° with 380 ul of 0.3 M NaoH and
30   30 mM EDTA, neutralized with 400 ul of 2 M NH$_4$ Ac and 400
ul was applied in duplicate to BA85 nitrocellulose
filters using a Minifold II Slot Blot® apparatus
(Schleicher and Schuell, Dassel, FGR). The
filters were baked for 2 hours at 80° and prehybridized
35   for 2 hours at 45° in a solution of 6 x SSC, 1 x
Denhart's solution and 0.1% SDS. They were then
separated and hybridized in separate containers with 300

ng of either oligonucleotide probe 17-C or 15-T, in 30-50 ml of 6 x SSC, 1 x Denharts solution and 0.1% SDS at 45° for 4 hours. Oligonucleotide probes were end labelled with ^{32}P using [^{32}P]ATP and T$_4$ polynucleotide kinase

5   [Maniatis et al (1982) *supra*]. Filters were washed with shaking 6 times with 50 ml of 6 x SSC and 0.1% SDS over the course of 30 min and with 500 ml of the same solution at 61° (probe 17-C) and 55° (probe 15-T) for 20 min. They were then exposed to x-ray film for 1 to 14 hours

10   and diagnosis was made on the basis of differential probe binding. Diagnoses for many of the animals were compared with diagnoses made by standard techniques as outlined below.

     Pietrain and Yorkshire swine were obtained from

15   closed herds inbred for more than ten years, and maintained at the Ontario Veterinary College, University of Guelph [O'Brien (1990) *supra*; O'Brien (1991) *supra* and MacLennan (1983) *supra*]. All of the Pietrain and none of the Yorkshire breeding stock in these herds were shown to

20   be positive for the halothane challenge test [O'Brien (1990) *supra* and Sanger 1977) *supra*] or to produce offspring that were positive for this test. The frequency of halothane reactors in Ontario Yorkshire swine from which our herd was derived was 0.0078 [Miller

25   (1988) *supra*]. The test apparently detects more than 90% of MH homozygotes and less than 25% of MH heterozygotes. Based on this test, our Pietrain swine were considered to be homozygous for the MH defect, whereas our Yorkshire herd was considered to be free of homozygotes for the MH

30   defect. MH susceptibility status was verified by the halothane and caffeine contracture tests [O'Brien (1990) *supra*] and O'Brien (1990) *supra*], performed on *gracilis* muscle from 11 individuals from our Yorkshire herd and 25 of our Pietrains. If the caffeine concentration

35   producing a 1 gram increase in tension (CSC) was less than 4 mM if the muscle developed more than 0.5 g tension with exposure to 5% halothane, swine were considered to

be MH-susceptible. If the CSC was greater than 7 mM and halothane produced less than 0.2 g increase in tension, swine were considered to be normal. All pietrain swine were considered to be positive, 9 Yorkshires were

5   considered to be positive and 2 Yorkshires with CSC between 5 and 6 were considered to be possible MH heterozygotes. The gene frequency predicted by contracture test results was identical to that predicted by halothane-testing of Ontario Yorkshire swine [Miller

10   (1988) *supra*]. Microassays for hypersensitivity of the Ca^{2+}-release channel were performed on passively-loaded terminal cisternae-enriched preparation isolated from *longissimus dorsi* muscle of six MH Pietrain and six normal Yorkshire swine [O'Brien (1986) *supra* and O'Brien

15   (1990) *supra*]. Compared to normal Yorkshires Ca^{2+}-release for the MH Pietrain swine occurred at tenfold lower concentration of channel agonist. At similar concentrations of agonist, Ca^{2+}-release occurred at three fold faster and in fourfold larger amounts. In order to

20   obtain heterozygotes for the MH gene, Pietrain were cross-bred to halothane-resistant Yorkshire swine, and halothane-reactive Landrace were cross-bred to halothane-resistant Landrace swine. Swine derived from the latter cross were shown to be halothane-resistant, although

25   parentage studies indicated that they were carriers of the MH gene. The DNA-probe test was also performed on the halothane-reactive offspring derived from cross-breeding halothane-reactive Landrace. These were considered to be likely homozygotes, but a heterozygous

30   state could not be ruled out. Swine that were either homozygous or heterozygous for the MH gene were considered to be MHS. Blood was also collected and analyzed from randomly selected swine from two Duroc herds that had each produce one or two halothane-reactive

35   pigs.

Example 3

Genomic DNA was isolated from fresh pig blood or frozen pig muscle [S.A. Miller, D.D. Dykes, H.F. Polesky, Nucleic Acids Res. 16, 1215 (1988)]. For analysis of the

5   nucleotide 1843 mutation, a 74 bp sequence was amplified with 100 ng of the forward primer 5'-GTTCCCTGTGTGTGTGCAATGGTG-3', which corresponds to porcine RYR1 cDNA nucleotides 1811 to 1834 and 100 ng of the reverse primer 5'-ATCTCTAGAGCCAGGGAGCAAGTTCTC

10   AGTAAT-3' in which the last 24 nucleotides were complementary to RYRI cDNA nucleotides 1861 to 1884. For analysis of the Ban II polymorphism at nucleotide 4332 a 160 bp sequence was PCR amplified with the forward primer 5'-TACTATTACTCGGTGAGGGTCTTC-3 which corresponds to RYRI

15   cDNA nucleotides 4297 to 4320 and the reverse primer 5' TGCTGTGGATGTTGCCCTGCTCAT-3' which is complementary to RYR1 cDNA nucleotides 4433 to 4456. For analysis of the Rsa I polymorphism at nucleotide 13879 a sequence of 227 bp was PCR amplified with the forward primer

20   5'-ATGACATGGAGGGTTCAGCAGCCG-3', which corresponds to RYR1 cDNA nucleotides 13763 to 13786 and the reverse primer 5'-CTTGAGGCAGTTGTAGCCTATGAT-3' which is complementary to RYR1 cDNA nucleotides 13966 to 13989. The primers were annealed at 52° for 2 min, extended at 72° for 3 min and

25   denatured at 95° for 40 sec for 35 cycles, with a final cycle of 7 min for extension in a DNA thermal cycler (Perkin-Elmer Cetus). The Mg^{2+} concentration was 1.5 mM for amplification of the mutant sequence and 1 mM for amplification of the Ban II and Rsa I sequences in the

30   PCR reaction buffer provided by Perkin-Elmer Cetus. For hybridization analysis of the MH mutation, 50-100 ng of DNA were applied in duplicate to BA85 nitrocellulose filters using a Minifold II Slot Blot apparatus (Schleicher and Schuell, Dassel, FRG) according to the

35   manufacturer. Prehybridization and hybridization with the 17 nucleotide sequence 5'-TGGCCGTGCGCTCCAAC-3' (probe 17-C) and the 15 nucleotide sequence

5'-GGCCGTGTGCTCCAA-3' (probe 15-T), end labelled with ^{22}P,
were at 45° in 6 x SSC, 1 x Denhart's solution and 0.1%
SDS.   Filters were washed with shaking six times with 50
ml of 6 x SSC and 0.1% SDS over the course of 30

5   min and once with 500 ml of the same solution at 61°
(probe 17-C) and 55° (prob: 15-T) for 20 min.   After
exposure to x-ray film, diagnosis was made on the basis
of differential probe binding.   Digestion of the PCR
products with Hin PI, Ban II or Rsa I were carried out

10   using standard protocols.   Sequence analysis of 3-8
subcloned 74 bp PCR products from each of heterozygous
Yorkshire, Poland China, Duroc and Landrace individuals
confirmed the presence of the C at position 1843 to T in
each breed and its absence in a homozygous normal

15   Hampshire animal.

MHS Pietrain and MHN Yorkshire were crossbred to
produce heterozygotes.   All MHN Yorkshires used in these
experiments had been shown to be negative for the
halothane challenge test and to produce negative

20   offspring.   In the halothane challenge test, 2 to 3 month
old pigs are physically restrained and forced to inhale
3% to 5% halothane in oxygen for several minutes [O'Brien
et al (1990) *supra*].   Those developing extensor muscle
rigidity are considered MHS.   This test detects more than

25   90% of MH homozygotes and less than 25% of MH
heterozygotes.   MHS Landrace and MHN Landrace were also
cross-bred to produce heterozygotes.   All other swine
samples were obtained from local herds.   Thirteen
Landrace swine were studied; eight were positive for the

30   halothane challenge test and five were offspring of swing
positive for the halothane challenge test.   Blood was
collected from randomly selected swine from two Duroc
herds that had each produced pigs that had a positive
halothane test.   All Pietrain swine and no Yorkshire

35   swine in these herds were positive for the halothane
challenge test.   Susceptibility status of 25 Pietrain and
15 Yorkshire swine was confirmed by the caffeine-

64

/halothane contracture test [O'Brien et al (1990) *supra* and Miller et al (1988) *supra*], in which muscle was excised, placed in oxygenated physiological saline and connected to a force-displacement strain gauge.

5   Isometric tension was recorded on a polygraph during exposure to progressively increasing amounts of caffeine or to 5% halothane. If the caffeine concentration producing a 1 gram increase in tension was less than 4 mM or if the muscle developed more than 0.5 g tension with

10   exposure to halothane swine were considered to be MHS. All Pietrain pigs were positive for these tests, but 13 of 15 Yorkshire pigs developed 1 g contracture only at concentrations greater than 7mM, while two had values intermediate to those of the other Yorkshires and the

15   Pietrains. These were considered to be possible MH heterozygotes.

Swine were also tested for Ca^{2+} release channel activity [O'Brien et al (1990) *supra*]. A terminal cisternae-enriched fraction was passively loaded with

20    $^{45}Ca^{2+}$ and rates and amounts of release were determined at different concentrations of caffeine using a positive-pressure ultrafiltration system and scintillometry. Ca^{2+} release for the MHS Pietrain swine occurred at 10-fold lower concentrations of channel agonist, at threefold

25   faster rates and in a fourfold larger amounts than for the MHN swine.

Example 4 PREPARATION OF BLOOD SAMPLES FOR PCR

Detection of C1843 to T mutation is detected in accordance with the following by digestion of amplified

30   DNA with HgiAl restriction endonuclease.

This procedure dissolves the cytoplasmic membrane and pellets nuclei. Therefore cytoplasmic DNA is lost. Should yield about 20 ug of DNA. The procedure depends on osmotic lysis of cells & pelleting of nuclei & cell

35   debris. Hemoglobin released from RBC's is washed away in several pelleting & washing steps. The hemoglobin is inhibitory of PCR at concentrations as low as 0.8 uM hematin or at less than 1% blood.

1)   Mix 500 uL blood with 500 uL "lysis buffer"
      (see below) in a 1.5 mL Eppendorf
      microcentrifuge tube.

5   2)   Centrifuge 13,000 g for 10 sec using Eppendorf
            table-top microcentrifuge Model # 5415

3)   Remove supernatant with pipet and resuspend
      pellet using vortex mixer in 1 mL "lysis
10   buffer".

4)   Repeat steps 2 and 3 twice. Repeat an
      additional time if the pellet is still red
      (This happens with an occasional sample).

15

5)   Centrifuge 13,000 g for 20 sec, remove
      supernatant and resuspend in 200 uL of "PCR
      buffer with nonionic detergents and Proteinase
      K". This should yield a genomic DNA
20   concentration of 0.1 ug/uL.

6)   Incubate at 50 to 60°C for 1 hour.

7)   Incubate at 95° C for 10 min to inactivate
25   Proteinase K to prevent its degradation of
      enzymes used in PCR.

8)   Store frozen if required at -85°C.

30   9)   Up to 50% of the PCR medium may be this
            digested lysate.
      "Lysis buffer" (from Buffone, GJ & Darlington GJ (1985)
      Clin Chem 30(1):164-5)

35   320 mM sucrose [MW = 342.3 g/mol; use 109.54 g/L]
      10 mM Tris-HCl (pH 7.5) [MW = 121.14 g/mol; use 1.2114
      g/L]

5 mM MgCl$_2$ [MW = 95.21 g/mol; use 0.4761 g/L]

1% (w/v) Triton X-100; use 10 mL/L

"PCR buffer with nonionic detergents & proteinase K"

50 mM KCl

5   10 mM Tris-HCl (pH 8.3)

2.5 mM MgCl$_2$

0.1 mg/mL of gelatin

0.45% (w/v) NP40

0.45% (w/v) Tween 20

10   Autoclave & store frozen. When ready to use, thaw &

add 6 uL of 10 mg/mL Proteinase K (in water) per 200 uL

of PCR buffer solution.

PCR AMPLIFICATION

1).   Prepare the reagents (1 through 10) in one large

15   "mastermix" with enough volume to perform PCR for all

samples and standards; then add this mastermix to each

PCR tube.

Order of addition of reagents:

20   1.   sterile distilled water   6.775 uL

(brings final vol to 25 uL when consider 11.5 uL added

previously)

2.   10x PCR buffer II   2.5   uL

3.   10 mM dATP   0.5   uL

25   4.   10 mM dCTP   0.5   uL

5.   10 mM dGTP   0.5   uL

6.   10 mM dTTP   0.5   uL

7.   Primer FL17F(orward)   0.20   uL

8.   Primer FL17R(everse)   0.20   uL

30   9.   25 mM MgCl$_2$ 1.5   uL

10.   AmpliTaq DNA polymerase   0.125   uL

(5 U/uL; comes in solution at this conc)

-preparing a mastermix reduces the vol of work &

improves precision & reduces intersample variability

35   -the same pipette tip can be used to add the

mastermix but separate autoclaved pipette tips must be

used for each of the genomic DNA samples in order to prevent cross-contamination

11. genomic DNA   1 uL

    (conc is approx 0.2 ug/uL)

2) After addition of sample's genomic DNA to mastermix then cap the reaction tubes.

3) Start 2-step PCR

    Place tray of tubes in sample block of PCR machine. Access main menu of PCR machine and use the following parameters; 1: 94°C for 10 sec, 67°C for 80 sec, 30 cycles, 25 uL reaction volume

4. Notes:

    -kits can be purchased from Perkin Elmer Cetus with all reagents necessary for PCR: we have been using the "GeneAmp PCR Core Reagent" kit cat # N808-0009 which has the dNTP's, polymerase, 10x PCR buffer, $MgCl_2$

    -add polymerase last since it is heat-sensitive

    -deoxynucleotides are dissolved in glass-distilled water & titrated to pH 7.0 with NaOH; their concentrations must be balanced since if one is higher then the others the polymerase may incorporate them, slow down & terminate

    -high concentrations of primer (>0.2 uM) can lead to amplification of non-target segments (if so try reducing primer levels)

    -for a standard 100 uL reaction containing 2.5 units of Taq polymerase, substrate excess conditions begin to occur around 1 ug DNA (3 nmol of dNMP)

    -forward primer is 5'-TCCAGTTTGCCACAGGTCCTACCA-3'

    -reverse primer is 5'-TTCACCGGAGTGGAGTCTCTGAGT-3'

    -Note that the 3' ends of the primers overlap with the result that "autoprimerization" occurs leading to dimerization and "dimer-dimerization" ie extra bands corresponding to 2x MW of primers and 4x MW of primers;

this artefact can be eliminated by increasing or
decreasing the length of the primers by 1 nucleotide

-100 ng of ea primer is used per 100 uL; primer conc
between 0.1 & 0.5 uM are generally optimal; higher primer
5   concentrations may promote mispriming & accumulation of
nonspecific product & may increase the probability of
generating a template-independent ar:ifact termed primer-
dimer; nonspecific products & primer-dimer artifacts are
themselves substrates for PCR & compete with the desired
10   product for enzyme, dNTP's & primers, resulting in a
lower yield of the desired product

-for old PCR machine (DNA Thermal Cycler from Perkin
Elmer Cetus) we have successfully used 94 C for 60 sec,
52 C for 120 sec & 72 C for 180 sec
15   -for 3 step PCR we have successfully used: 94 C for
10   sec for template melting; 53 C for 120 sec for
annealing, and 72 C for 60 sec for extending; 35 cycles
-the ramp time is the time taken to change from one
temperature to another.
20   <u>10x PCR buffer II</u>

500 mM KCl
100 mM Tris-HCl
pH 8.3 at room temp
25   Provides preferred pH & ionic strength for PCR.
Gelatin free. Component of Perkin-Elmer Cetus GeneAmp PCR
Core Reagent kit
<u>AmpliTaq DNA Polymerase</u>
-purchased from Perkin Elmer Cetus
30   -this is the recombinant form of Taq DNA polymerase; it
is obtained by expressing the Taq DNA polymerase gene in
an E coli host
5).   3 phases to PCR amplification:
1) template melting:
35   -here the doubled-stranded DNA is heat-denatured;
typical denaturation conditions are 95 C for 30 sec or 97
for 15 sec;

-AmpliTaq DNA polymerase has a half-life < 35 min at > 95
C; 5 min at 97.5 C but > 2h at 92.5 C;
-it is very important in the early cycles to be sure to
completely melt the template DNA;

5   -incomplete denaturation allows the DNA to "snap back" &
thus reduces product yield whereas denaturation steps
that are too high &/or too long lead to unnecessary loss
of enzyme activity;
-when genomic DNA is used as the starting template,

10   melting at 9·7 C for the first few cycles will ensure
single stranded templates for the PCR reaction; the
melting temperature can be reduced for the later cycles
because the smaller PCR product usually melts completely
at a lower temperature (unless PCR product is excessively

15   G+C rich) than the starting genomic DNA.
          2) annealing:
-here the 2 primers complementary to the 3' boundaries of
the target segments are annealed at a low temperature ;
-higher annealing temperatures (45 - 65 C) generally

20   results in a much more specific product.
          3) extension:
-here the annealed primers are extended by AmpliTaq DNA
polymerase at an intermediate temperature; extension is
from 5' to 3';

25   -AmpliTaq DNA polymerase extends at 2000 - 4000 bases per
minute at 70 - 80 C; thus actually need much less than
one min for extending 1 kilobase;
-using a 2 temperature cycle decreases cycling time while
still maintaining specificity & the level of

30   amplification;
-in 2 step PCR the extension time is eliminated
completely for small target sequences since the
polymerase retains significant activities at lower
temperatures & complete extension occurs during the

35   thermal transition from annealing to denaturation

          4) cycles:

-the optimum number of cycles depends mainly on the
starting conc of target DNA when other parameters are
optimized; a common mistake is to execute too many
cycles; too many cycles can increase the amount &
5   complexity of nonspecific background products whereas too
few cycles gives low yield; use 25 - 30 cycles for
300,000 target molecules, 30 - 35 for 15,000 targets, 35
to 40 for 1,000 targets & 40 - 45 for 50 targets;
-pigs normally have at least 10,000 WBC's per uL of
10   blood; assuming each has a copy of the RYR gene and that
yield is 1% (very conservative) then for a 500 uL blood
sample of which 10% of its lysate is used for PCR, there
should be at least 5,000 targets, thus 35 cycles is
appropriate; if yield is higher (say 10%, not unlikely)
15   then 30 cycles may suffice.

6) PCR Product Digestion (in a total volume of 33.3 uL)

1)   10x NE buffer 3 (supplied with re)   2.83
20   1 M NaCl
         500 mM Tris-HCl
         100 mM $MgCl_2$
         10 mM DTT
         pH 7.9 at 25 C)

25

2)   HgiA1 (5 U/uL)   0.5
     New Eng BioLabs cat # 170L stored at -85 C
                 1 U = 1 ug lambda DNA digested at 37 C in 50
                 uL assay buffer in 1 hour
30   3)   PCR product   25
     -add the buffer and HgiA1 directly to the PCR
     microreaction tubes
     -then digest at 37 C for 1 in the PCR machine
     7)   AGE: Gel Preparation for 1 large gel
35   1) 1x TBE buffer   350 mL
         89 mM Tris base
         89 mM boric acid

71

        2 mM EDTA

        pH 8.0

   2) NuSieve 3:1 Agarose   9 g

   3) 10 mg/mL ethidium bromide   35 uL

5   of 10 mg/mL (final conc of 0.01%)

        -bring to boil on hot plate with stirrer

        -cool to 55°C then pour into tray and allow to gel which takes approx 30 min

        -when gelled if not going to use for a while cover

10 with a thin film of TBE

   8) <u>AGE: Electrophoresis</u>

        -add 5 uL of loading buffer (see below) to each PCR digest to bring final volume to 25 uL

15   -add entire volume (33.3 uL) to each well of gel

        -use up to 3 of the 36-toothed combs for the large horizontal electrophoresis apparatus (Hoefer Scientific Instruments; HE100 SuperSub Horizontal Unit)

        -add 10 uL for standard DNA digest for both large

20 and small gels

        -perform constant voltage electrophoresis:

            minigel 150 volts for 1 h

            large gel 250 volts for 1.5 h with cold (4°C) water circulation

25   -for controls run: homozygous PSS pig (Pietrain)

            homozygous normal pig (Yorkshire)

            heterozygous pig (from previous diagnosis)

            uncut PCR product (from fresh sample)

            DNA std

30   -run a set of controls for each comb

   Loading buffer PreparationFor 50 mL

   25% Ficoll 400   12.5 g

   25 mM EDTA   1.25 mL of 1 M EDTA

35 1% SDS   0.5 g

   1 mg% Bromphenol blue   1 mg Sigma cat #B-6131

● ●

9) AGE: RFLP Visualization & Photography

-we find the best procedure is to destain for 1 hour
in warm tap water and then photograph; this gives best
resolution of banding; too prolonged destaining results
5   in the bands being less visible;

-stain with 0.1 mg % ethidium bromide in water (20
uL of 10 mg/mL in 200 mL water) for 15 min; some
protocols (eg HSI SuperSub manual) recommend staining
with 50 mg% for 10 min; pour the stain into the gel tray;
10   -rinse 2 times in distilled water; 15 min is
recommended for each rinse; we have found that rinsing
for 1 h removes most of interfering background
fluorescence;

-DNA is visualized by fluorescence of intercalated
15   ethidium bromide which absorbs light at 260, 302 or 360
nm & emits at 590 nm; lower illumination wavelengths give
a higher sensitivity but also increase the risk of
damaging the DNA molecule; 302 is recommended for high
sensitivity without too much damage to the DNA fragments;
20   the dye has poor affinity for single stranded DNA;

-visualize banding pattern on transilluminator
(Hoefer Scientific Instruments; Mighty Bright model)
inside of "The Benchtop Darkroom";

-the running trays of the SuperSub are UV
25   transparent; trays can be placed directly on top of the
illuminator to reveal fluorscent bands; however maximum
fluorescence is seen when the gel is placed directly on
the transilluminator surface; with photography it is
possible to detect as little as 1 - 10 ng DNA
30   -photograph with Polaroid camera;

10)   Banding patterns:
1)   standard: phi X-174-RF DNA from Hae III digest;
concentration is 500 ug/mL; stored at -85 C in 10 mM
35   tris-HCl (pH 7.5) with 1 mM EDTA; phi X-174-RF DNA was
isolated from E coli infected with phi X am3cs70 and
digested to completion with Hae III; purchase from

Pharmacia: cat # 27-4044; there are 11 fragments: #1 -
1358; 2 - 1078; 3 - 872; 4 - 603; 5 - 310; 6b - 281; 61 -
271; 7 - 234; 8 - 194; 9 - 118; 10 - 72

      2)   homozygous PSS: bright band at 358 bp & faint

5   bands at 166 bp, 135 bp (faintest band)

      3)   homozygous normal: bright band at 524 bp with
faint band at 135 bp

      4)   heterozygote: bright bands at both 524 and 358
bp with lightly fluorescing bands at both 166 (faintest)

10   & 135

      5)   uncut PCR product: 659 bp

      6)   primer dimer dimer: 96 bp

      7)   primer dimer: 48 bp

      8)   primer: 24 bp

15

CLAIMS

1.   A purified DNA molecule comprising a DNA sequence of approximately 15.1 kb coding for normal or mutant RYR1 protein having a molecular weight of approximately

5   564,740 daltons, said DNA molecule having an endonuclease restriction map of Figure 1.

2.   A purified DNA molecule comprising the DNA sequence of Figure 2 for amino acid residue positions 1 through

10   5035.

3.   A purified DNA molecule comprising at least 12 nucleotides selected from the group of nucleotides consisting of DNA nucleotides for amino acid residues

15   extending from amino acid residue positions 1 to 5035 of Figure 2.

4.   A purified DNA molecule comprising a DNA sequence encoding normal or mutant RYR1 protein having amino acid

20   residue positions 1 through 5035 of Figure 2.

5.   A purified DNA molecule comprising at least 18 nucleotides encoding a fragment of normal or mutant RYR1 protein, said fragment being selected from 6 sequential

25   amino acid residues of said amino acid sequence having residue positions 1 through 5035 of Figure 2.

6.   A purified DNA molecule selected from the group consisting of 1 or more fragments selected from intron

30   sequences of Figure 6, each of said fragments being at least 12 bases long.

7.   A purified DNA probe comprising a DNA sequence of claim 3.

35

8.   A purified DNA probe comprising a DNA sequence of claim 5.

●   ●

9.   A recombinant cloning vector comprising a DNA
molecule of claim 1, 2, 3, 4, 5, or 6.

10.   A host transformed with a recombinant cloning vector
5   comprising a DNA molecules of claims 1, 2, 3, 4, 5, or 6.

11.   A host of claim 10 wherein said host is a
prokaryotic or eucaryotic cell, said DNA molecule being
operatively linked to an expression control sequence so
10   that said RYR1 protein is expressed, said expression
control sequence being selected from the group consisting
of sequences that control the expression of genes of
prokaryotic or eucaryotic cells.

15   12.   A method for screening a pig to determine if said
pig is susceptible to malignant hyperthermia comprising:
        i)   providing a biological sample which was removed
        from said pig to be screened, and
        ii)   conducting a biological assay to determine
20   presence in said biological sample of at least a
        member selected from the group consisting of:
        a)   mutant RYR1 gene having a mutation C1843 to T
               1843 in the DNA sequence of Figure 2
        b)   mutant RYR1 protein having an arginine 615
25   mutation to cysteine 615 in the amino acid
               sequence of Figure 2
        c)   one of more of 17 DNA polymorphisms identified
               in the DNA sequence of Figure 2;
        d)   one or more unique endonuclease restriction
30   sites of corresponding DNA sequence of Figure 1
               or Figure 6.

13.   A method of claim 12 wherein said biological sample
includes at least part of a genome of said pig, said part
35   including at least a DNA fragment from the normal or
mutant RYR1 gene represented by said DNA sequence of
Figure 2.

14.   A method of claim 13 wherein said assay comprises a
DNA hybridization assay in which a labelled DNA probe of
claim 7 or 8 is used.

5   15.   A method of claim 13 wherein said assay comprises a
DN. hybridization assay in which a labelled DNA probe is
used, said probe having a sequence including either a
T1843 or C1843 base in the DNA sequence of Figure 2.

10   16.   A method of claim 13 wherein said assay comprises a
one step, allele specific, PCR-based diagnosis of the DNA
sequence in said biological sample, said assay comprising
a PCR amplification of DNA in said biological sample by
use of predetermined forward and reverse primer
15   sequences, said selected forward or reverse primer
including a C1843 base for specific detection of a normal
RYR1 gene and said selected forward or reverse primer
including a T1843 base for specific detection of a mutant
RYR1 gene.

20

17.   A method of claim 16 wherein said selected forward
or reverse primer includes all of any part or one of the
sequences (17-c) 5'-TGGCCGTGCGCTCCAAC-3' or (15-t) 5'-
GGCCGTGTGCTCCAA-3'.

25

18.   A method of claim 13 wherein said assay comprises
restriction endonuclease digestion of amplified DNA
fragments in said biological sample, said digestion
comprising use of an enzyme selected from the group of
30   enzymes for digesting sites represented by HinP1 and
HgiA, said HinP1 site being indicative of normal RYR1
gene and said HgiA1 site being indicative of mutant RYR1
gene.

35   19.   A method of claim 15 wherein said DNA probes are
selected from the group consisting of (17-c) 5'-
TGGCCGTGCGCTCCAAC-3' and (15-t) 5'-GGCCGTGTGCTCCAA-3'.

20.   A method of claim 13 wherein RFLP analysis is conducted on said DNA in said biological sample to determine presence of said one or more of said 17 polymorphisms.

5

21.   A substantially pure RYR1 protein having a molecular weight of approximately 564,740 daltons and having an amino acid sequence corresponding to the amino acid sequence of Figure 2.

10

22.   RYR1 protein of claim 21 wherein mutant RYR1 protein comprises a mutation of arginine 615 to cysteine 615.

23.   RYR1 protein purified from culture of a host

15   transformed with a recombinant cloning vector comprising a DNA molecule of claim 1, 2, 3, 4, 5, or 6.

24.   A purified protein fragment comprising at least six amino acid residues of said sequence of claim 21.

20

25.   An antibody specific for said RYR1 protein of claim 21.

26.   An antibody specific for an antigenic determinant of

25   said RYR1 protein of claim 21.

27.   An antibody specific for said protein fragment of claim 24.

30   28.   An antibody specific for normal RYR1 protein of claim 21 having arginine at residue position 615.

29.   An antibody specific for mutant RYR1 protein of claim 21 having cysteine at residue position 615.

35

30.   A method of claim 12 wherein an antibody of claim
25, 26, 27 or 29 or functional fragment thereof is used
in determining presence of said mutant protein.

5   31.   A method of claim 12 wherein an antibody of claim
25, 26, 27 or 29 or   functional fragment thereof is used
in determining presence of said mutant protein, said
antibody being a polyclonal antibody.

10   32.   A method of claim 12 wherein an antibody of claim
25, 26, 27 or 29 or a functional fragment thereof is used
in determining presence of said mutant protein, said
antibody being a monoclonal antibody.

15   33.   A method of claim 12 wherein an antibody of claim
25, 26, 27 or 29 or a functional fragment thereof is used
in determining presence of said mutant protein, said
antibody being labelled with a label selected from the
group consisting of a radiolabel, fluorescent matrix, and
20   an enzyme.

34.   An assay kit suitable for use in screening pigs to
determine susceptibility to malignant hyperthermia due to
presence of a mutant RYR1 gene in a pig genome, said kit
25   comprising:
i)   oligonucleotide priming sequences for PCR
amplification of selected DNA sequence in said RYR1 gene,
said DNA sequence being selected from sequences either
spanning or to either side of DNA mutation 1843 of Figure
30   2;
ii)   optionally oligonucleotide probes which hybridize
with either normal DNA at residue position 1843 of Figure
2 or mutant DNA at residue position 1843 of Figure 2; and
iii) reagent means for detecting hybridization of said
35   probes to said RYR1 DNA in the region of residue 1843 or
hydridization of said forward or reverse primer to
residue 1843.

35.   An assay kit of claim 34 wherein said
oligonucleotide priming sequences are selected from the
group consisting of:

    i)   forward primer nucleotides 1771 to 1794 of
Figure 2;

    ii)   reverse primer nucleotides 1929 to 1952 of
Figure 2;

    iii) forward primer sequence
        5'-TCCAGTTTGCCACAGGTCCTACCA-3'

    iv)   reverse primer sequence
        5'-TTCACCGGAGTGGAGTCTCTGAGT-3'

    v)   forward primer sequence 17-c; and

    vi)   forward primer sequence 15-t.

36.   An assay kit suitable for use in screening pigs to
determine susceptibility to malignant hyperthermia due to
presence of mutant RYR1 gene in a pig's genome, said kit
comprising:

    i)   an antibody which specifically binds to mutant
RYR1 protein expressed by said mutant RYR1 gene
having a sequence corresponding to the cDNA of
Figure 2;

    ii)   reagent means for detecting binding of said
antibody to said RYR1 protein; and

    iii) said antibody and reagent means being present
in amounts effective to perform the immunoassay.

37.   An assay kit of claim 35 wherein said antibody is
selected from antibodies defined in claims 25, 26, 27, 28
or 29.

38.   An assay kit of claim 37 wherein said antibody is a
monoclonal antibody specific to the cysteine amino acid
mutant substitution at residue position 615.

1/13

FIG.1.

FIG.2.

FIG 2 con't

SUBSTITUTE SHEET

FIG.2 con't.

CTGGAGCTGTCCGAGCGCTTGGACTTGCACCAGTTCCACTCGCACACCCTCTACCGCGTTGCGCTCTGGGCAACAACCGCGTGCGCCGTCTGCACCCGTCTGCAGCGAC   5100
L E L S E R L D L Q Q F H S H T L R L Y R A V C A L G N N R V A H A L C S H V D Q A Q L L H A L E D   1700

GCGCACCTGCCCCCGGGCCCTCTGCGCGGCCAGGTTACTACGACCTCCTCATCAGCATCCACCTGGAAAGCGCCTGCCGCAGCCGCCGCCATCACTCTCTTCCCGCCT   5250
A H L P G P L R A G Y Y D L L I S I H L E S A C R S R R S M L S E Y I V P L T P E T R A I T L F P P   1750

GGAAAAGGACGGAAAACGGTCCCCCGCACGGCCTGCCCGGTGTCGGCGTCACCTCGCGTGAGCCTCGCGGTGTTGGCAGCCCGCCGGAGCCCGGCCCGCTCAGC   5400
G K R T E N G P R R H G L P G V G V T T S L R P P H H F S A P C F V A A L P A V G A A A E A P A R L S   1800

CCCAGCCATCCCTCTGGAGGCTGGGGGACAAAGCACTAAGAATCCTGGGGAGGCGGTACGGACGCGGGTGGGCAGCACGCACGCGACCCTGTCTCGGGAGTTCGTGCCCGTGCTCAAGCTCGTCCACCTGCTG   5550
P S I P L E A L R D K A L R M L G E A V R D G G Q H A R D P V G G S V E F Q F V P V L K L V S T L L   1850

GTGATGGGCATCTTGGTGATGAAGACGTGAAGCAGATCTTGAAGCAGATATTCACTGACAGTATTCACTGACCCAGAAGATGAGAAGAAGAGGAGGATGAGGAGGAG   5700
V M G I F G D E D V K Q I L K M I E P E V F T E E E E E E E E E K E E D E E E E K E D E E E E   1900

GCAACGGGAGAAGGAGGATGAGGAGAAAAAGACAGCGAGACACCAGAAAAGAATACTTGGAGAAGGGCTGCTCCAGACTCGTGAAGTTACCAGATGTCTGTAATCTGTTGGAGTATTTCTGTGACCAA   5850
A R E K E D E E K E E E T A E G E K E E Y L E E G L L Q M K L P E S V K L Q M C N L L E Y F C D Q   1950

GAGCTGCAGCACGTGTGGAGTCCCGCAGCCTTTGCAGAACGCGACGGCATCCTCAGGCAACGAGGCAGCGTCCAACCATGACCGGCTGCCGAGACTGCCCGAGACTGCCGCGACTTCCGC   6000
E L Q H R V E S L A A F A E R Y V D K L Q A N Q R D R Y G I L H K A F T M T A A E T A R R T R E F R   2000

TCCCCACCCCAGGAGCAGATCAACATGCTATTGCACTTCAAAGATGGCGAGGGATGAGGAAGATTGCCTCTTCCTGATGAGATCCGGCAGGATTGGAATTCCATCAAGACCTGTTGACTCACTGTGAATCAGCTGGAGGGGGAG   6150
S P P Q E Q I N M L L H F K D G E D E D C P L P D E I R Q D L L E F H Q D L L T H C G I Q L E G E   2050

FIG.2 con't.

```
GAGAAGAACCAGAGGAGGAAGAAGGCCACCCTGGGCAGCCGCCTTGATGAGCCTCGTTGAGAGAAGGTGCGCTGGTGAAGATCCGGAGGAGCAGCCACCCCAGTCACTGCCAGGAGCTGGTGTCT   6300
 E  E  E  P  E  E  E  A  T  L  G  S  R  L  M  S  L  L  E  K  V  R  L  V  X  K  K  E  E  S  K  P  Q  S  L  Q  E  E  L  V  S          2100

CACACAGTGGTCGCGTGGGCCCAAGAGGACTTTGTGCAGAGCCCGGAGCTGGTACGGGCCATGTTCAGCCTGCTCCGCCTCCCGCCTACCACCATCTCCCCTCCTGTG                    6450
 H  T  V  V  R  W  A  Q  E  D  F  V  Q  S  P  E  L  V  R  A  H  F  S  L  L  R  Q  Y  D  G  L  G  E  L  L  R  A  L  P  R  A  Y  T  I  S  P  S  S  V   2150

GAGGACACCATGAGCCTACTCGAGTGCCTCGGCGTCACTGCTCATCGGCAGAAACCTCTTCTACCACACCGAACTTGATGCGG                                             6600
 E  D  T  M  S  L  L  E  C  L  G  Q  I  R  S  L  L  I  V  Q  M  G  P  Q  E  E  N  L  M  I  Q  S  I  G  N  I  H  N  N  K  V  F  Y  Q  H  P  N  L  M  R   2200
                                                                                                    A
GCACTGGGCATGCACGAGACAGTCATGGAGGTCATGGTGAATGTCGTCCTCGGCGGAGTCCGGCTTCCCAAGAGATCCGGCTTCTCTGCTACTTCTGCCGCATCAGCCGGCAGAACCAGCGGTCC   6750
 A  L  G  M  H  E  T  V  M  E  V  M  V  N  V  L  G  G  G  E  S  K  E  I  R  F  P  K  M  V  T  S  C  C  R  F  L  C  Y  F  C  R  I  S  R  Q  N  Q  R  S   2250

ATGTTTGACCACCTGAGCTACCTGCTCGAGAACAGTGGCATTGGCCTGGGCATGCAGGGCTCCCTGGATGTGGCCGCCGCGCTCCACGCCCCTGATGTGGCCGCCGCCTCCGTGATCGACAACAACGAGCTGGCTTTGGCCATTGCAGCACAACACGAGCTGGCAGAACAGGACCTGGAGAAGGTGTG  6900
 M  F  D  H  L  S  Y  L  L  E  N  S  G  I  G  L  G  M  Q  G  S  T  P  L  D  V  A  A  A  S  V  I  D  N  N  E  L  A  L  A  L  Q  E  Q  D  L  E  K  V  V   2300
```

FIG.2.con't.

FIG.2.con't. (nucleotide and amino acid sequence listing)

FIG.2 con't.

FIG.2 con't.

1811 GTTCCCTGTGTGTGCAATGGTGTGGCCGTGCGGCTCCAACCAAGATCTCATTACTGAGAACTTGCTGCCTGGC 1884

604 C S L C V C N G V A V R S N Q D L I T E N L L P G 628

(boxed: T / R / C)
(C above)
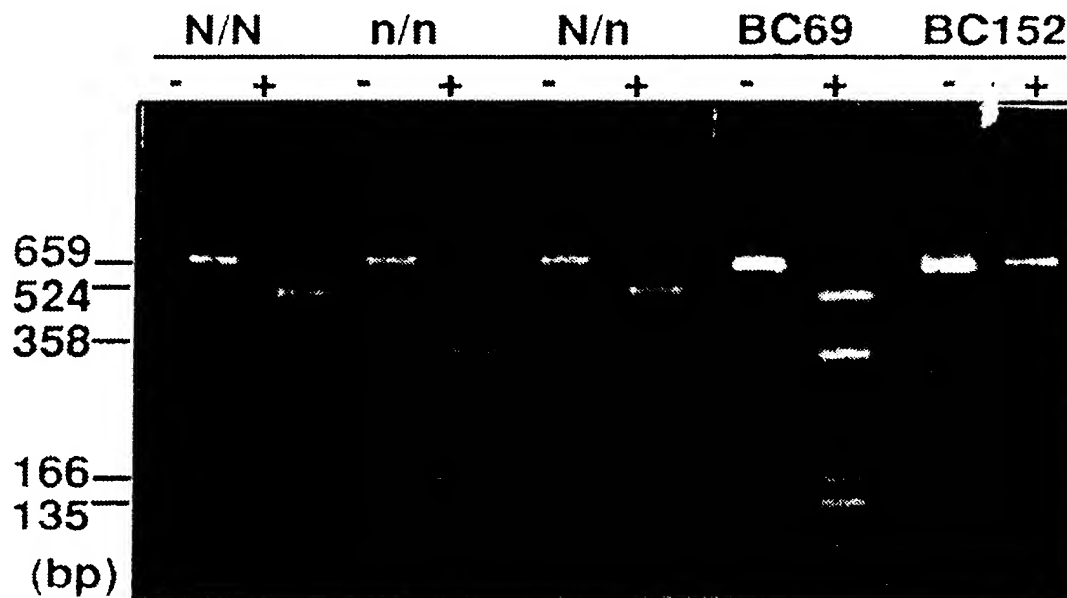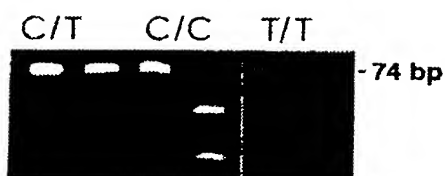
FIG. 4.

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FIG.8.

FIG.5B.

FIG.5C.

FIG.5D.

FIG.6.

```
GACAAGCATGGGAGGAGGAACCACAAGtgggcctctcatcccctcaattc........ctgatcttccccaccccctcttcactcttacctggactcctctg
 D  K  H  G  R  N  H  K

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tc................ ........agctcat............cctccatgtat......

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CCAAGATCTCATTACTGAGAACTTGCTCCCTGGCCGGGAGCTTCTGCTGCAGACAAACCTCATCAACTATGTCACCAGtctgtggccccccaaccttgac
 Q  D  L  I  T  E  N  L  L  P  G  R  E  L  L  L  Q  T  N  L  I  N  Y  V  T  S

ccagagcttagaacctccacccccgccccggtgaatggccctcctccgtcccccaccccgactaatgccagtc

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                                                             I  R  P  N  I  F  V  G
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SUBSTITUTE SHEET

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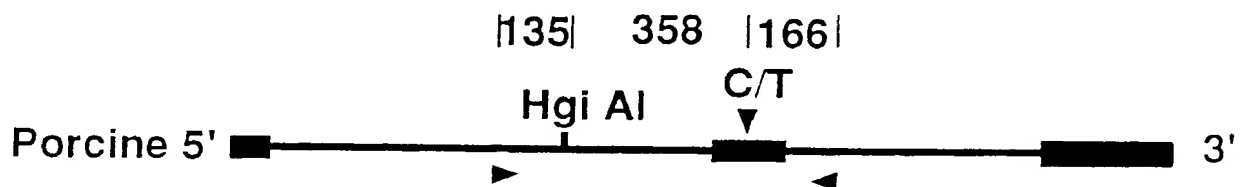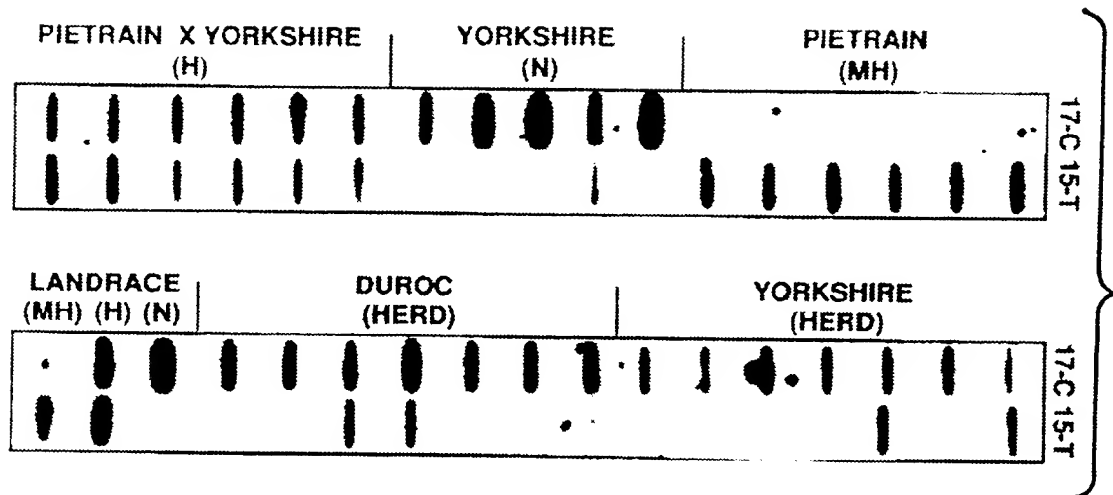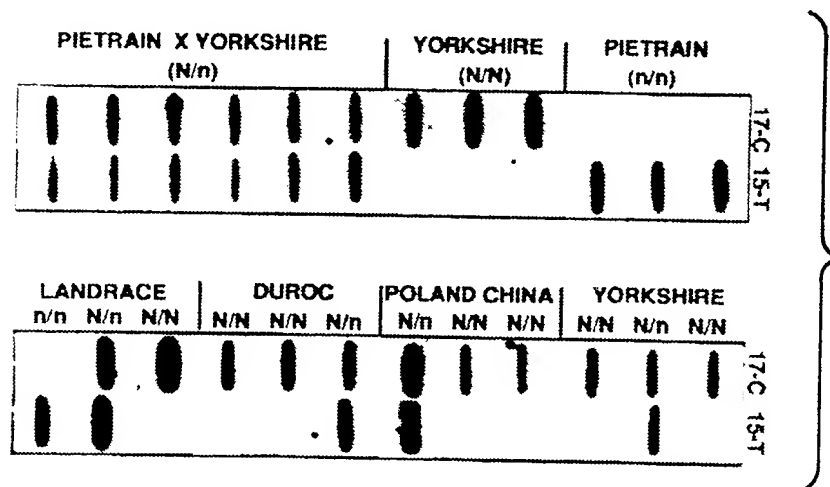**FIG. 7.**

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FIG.3.

FIG.5A.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Applic...

PCT/CA 91/00457

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; C12N15/12; G01N33/68; C07K13/00
C12P21/08; C07H21/04; //C12N15/63C07K15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q ; C07K ; C12N ; C12P

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 4, 5 February 1990, BALTIMORE US pages 2244 - 2256; F. ZORZATO ET AL.: 'Molecular cloning of cDNA encoding human and rabbit forms of the Ca2+ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum' cited in the application see the whole document	1-6

X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 23, 15 August 1990, BALTIMORE US pages 13472 - 13483; K. OTSU ET AL.: 'Molecular cloning of cDNA encoding the Ca2+ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum' cited in the application see abstract	1

	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 MARCH 1992	20. 03. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MOLINA GALAN_E

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	GENOMICS vol. 8, October 1990, SAN DIEGO US pages 243 - 248; I. HARBITZ ET AL: 'Assignment of the porcine calcium release channel gene, a candidate for the malignant hyperthermia locus, to the 6p11 - q21 segment of chromosome 6' cited in the application ---	
P,X	WO,A,9 104 328 (THE UNIVERSITY OF TORONTO INNOVATIONS FOUNDATION) 4 April 1991 see the whole document ---	1-15, 20-33, 36-38
P,X	SCIENCE. vol. 253, 26 July 1991, LANCASTER, PA US pages 448 - 451; J. FUJII ET AL: 'Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia' see the whole document ---	12-20

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9104328	04-04-91	AU-A- 6431390	18-04-91